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Stimulatory effect of endogenous and exogenous growth hormone secretagogues on isolated porcine somatotropes

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**Stimulatory effect of endogenous and exogenous growth hormone
secretagogues on isolated porcine somatotropes**

by

Aleksandra Glavaski

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY

Major: Veterinary Anatomy; Neuroscience

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For the Co-major Program

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For the Co-major Program

Dedicated to

my parents,

for encouraging and leading their children into intellectual pursuits

my sister Sonja,

for always being there when I needed her

my husband Milan

for his help and support

and son Luka.

for making everything worthwhile

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ABSTRACT

This dissertation is an effort to further define sites and cellular mechanisms of action of exogenous and endogenous GH secretagogues (GHS). The enclosed experiments have been performed on porcine somatotropes using an *in vitro* approach.

The first study looked at the ability of benzolactam GHS – L-692,585 (L-585) to stimulate Ca^{2+} transients and GH secretion from cultured porcine somatotropes. Perfusion application of L-585 dose dependently increased intracellular calcium ($[\text{Ca}^{2+}]_i$) in somatotropes. With concurrent reverse hemolytic plaque assay (RHPA) and $[\text{Ca}^{2+}]_i$ measurements, it was shown that the increase in $[\text{Ca}^{2+}]_i$ evoked by L-585 coincides with GH secretion. Incubation of cultured cells with L-585 increased the number and size of plaques around somatotropes, showing a stimulatory effect of L-585 on GH secretion.

The second study using atomic force microscopy demonstrated for the first time the presence of the fusion pores at the plasma membrane of isolated porcine somatotropes. In the resting somatotropes were found ‘pits’ containing several ‘depressions’, or fusion pores, with diameter of 100-200 nm. After stimulation of secretion with L-585 ‘depressions’ enlargement for about 40 % was observed. That ‘depressions’ are structures at plasma membrane of somatotropes involved in hormone secretion was confirmed by using gold-tagged GH-antibody. This study together with previous studies on pancreatic acinar cells suggests that fusion pores may be common structures for all secretory cells, and that they are the sites where following stimulation membrane-bound secretory vesicles transiently dock and fuse to release vesicular content.

The third study investigated the effects of an endogenous GHS - ghrelin on $[\text{Ca}^{2+}]_i$ in cultured porcine somatotropes. The application of ghrelin resulted with changes in $[\text{Ca}^{2+}]_i$ similar to those induced by L-585. Our experiments have shown that rapid increase in $[\text{Ca}^{2+}]_i$ induced both by L-585 and ghrelin originated from the intracellular stores, while the plateau phase resulted from somatotropes depolarization and extracellular calcium entry. Simultaneous application or application in succession of L-585 and ghrelin has revealed antagonistic effects between these two agonists that may result from competition at the receptor level and/or downstream from GHS-R.

Collectively, the results from these studies indicate that stimulatory effect of ghrelin and synthetic GHS on $[Ca^{2+}]_i$ in cultured porcine somatotropes closely parallel each other, in a manner that is consistent with an increase of GH secretion.

CHAPTER 1. GENERAL INTRODUCTION

Introduction

Neuroendocrine regulation of growth hormone (GH) secretion primarily involves reciprocal interactions between GH-releasing hormone (GHRH) and somatostatin (SS), exerting both stimulatory and inhibitory influences, respectively, on somatotropes. GH also participates in its own rhythmic secretion through feedback action on GHRH- and SS-neurons (Chan *et al.* 1996a).

A group of peptide and non-peptide mimetics, termed GH secretagogues (GHS), stimulate GH secretion via a mechanism distinct from GH-releasing hormone (GHRH) (Smith *et al.* 1996a, 1997). A receptor for GHS (GHS-R) was cloned from the pituitary of several species (Howard *et al.* 1996, McKee *et al.* 1997), and an initial mapping study identified numerous sites of expression of GHS-R in several hypothalamic nuclei, as well as in other brain structures (Guan *et al.* 1997). Recent cloning of ghrelin (Kojima *et al.* 1999), an endogenous ligand for GHS-R, has revealed that the GHS system is probably an additional physiological regulator of GH secretion, and it is likely to have global peripheral and central effects.

The focus of this dissertation is to elucidate site(s) and cellular mechanism(s) of action of GHS [ghrelin and L-692,585 (L-585)] on porcine somatotropes using an *in vitro* approach.

The effects of ghrelin and L-585 on GH release, changes in intracellular calcium ($\Delta[\text{Ca}^{2+}]_i$) and membrane structure associated with secretion in somatotropes were observed. This study is important for understanding the role of GH secretagogues in nutrient partitioning. Knowledge of the mechanisms by which ghrelin and other specific GHS stimulate GH secretion may ultimately be applied to management, nutrition and/or breeding/genetics approaches that optimize GH secretion. An understanding of mechanisms by which non-peptidyl GHS modulate GH secretion may be particularly significant because of their efficacy of oral administration, and possible clinical application.

Dissertation organization

This dissertation is composed of three journal papers. In the first and the third paper, the effects of L-585 and ghrelin on cultured porcine somatotropes were observed. All of the experimental and research work presented in these two papers was carried out by myself and under the guidance of Dr. Srdija Jeftinija and Dr. Lloyd L. Anderson. The first paper (Chapter 2) has been published in *Journal of Endocrinology* (175:625-636) and all material is reproduced by permission of the Society for Endocrinology. The second paper describes structure and dynamics of fusion pores in cultured porcine somatotropes. In that paper I am one of co-authors. I have cultured porcine pituitary cells, treated them with nonpetidyl GH secretagogue-L-585, and analyzed GH secretion stimulated by L-585 using reverse hemolytic plaque assay (RHPA), while atomic force microscopy (AFM) was done by our collaborators from Dr. Jena's laboratory. The second paper (Chapter 3) has been published in *Endocrinology* (143:1144-1148) and all material is reproduced by permission of publisher.

Each journal paper compromises a single chapter of the dissertation. The papers are preceded by a general introduction chapter containing a literature review, and followed by a general conclusion chapter consisting of a general discussion section and summary. The references are listed at the end of the chapter in which they are cited.

Literature review

Growth hormone

Growth hormone (GH), also called somatotropin, is polypeptide hormone synthesized and secreted by specific cells - somatotropes, of the anterior pituitary gland. Somatotropes are located mainly in two lateral wings of the anterior lobe. Those cells are usually medium size and spherical or oval, with a spherical, centrally located nucleus and with well-developed cytoplasm showing low electron density and containing slim rough endoplasmic reticulum cisternae and a conspicuous Golgi complex (Kovacs & Horvath 1985).

GH is an anabolic hormone responsible for postnatal growth, weight gain, and whole body nitrogen retention (Strobl & Thomas 1994). It causes growth of almost all tissues by

affecting protein, lipid and carbohydrate metabolism, cell multiplication and cell differentiation. Most of these growth effects of GH are achieved indirectly by stimulating production of small proteins called somatomedins that stimulate growth of bones and other tissues (Guyton 1991). Furthermore, there are some indications that GH also may be involved in regulation of sleep, reproduction, immune function, mental well being, and the aging process (Strobl & Thomas 1994).

Synthesis and release of GH are controlled by a number of factors, including hypothalamic and glandular hormones. GH is normally released from somatotropes in a circadian, pulsatile fashion; however, the basis of the pulsatile release of GH still remains unknown (Muller *et al.* 1999). Also unknown is whether the complex episodic pattern of GH secretion determines the effectiveness of GH on its target tissues. Studies using separate injection or infusion patterns confirmed that skeletal growth in rats is dependent on pulse frequency as well as amplitude for GH (Jansson *et al.* 1982, Clark *et al.* 1985), but continuous GH was much more effective than daily injections in reducing fat stores (Clark *et al.* 1996). The GH secretory pattern is sexually dimorphic (Eden 1979, Jansson *et al.* 1985). Sex-related differences in GH secretion patterns have been studied extensively in rats. Male rats exhibit low basal GH levels associated with the high amplitude and regular frequency pulses (Tannenbaub & Martin 1976), while female rats in contrast maintain elevated basal GH levels with irregular low amplitude pulses (Eden 1979, Clark *et al.* 1987). These differences are attributed to modulation of hypothalamic function by gonadal factors and higher levels of GH-releasing hormone (GHRH) and somatostatin (SS) expression in males as compared to females (Jansson *et al.* 1987, Argente *et al.* 1991, Lago *et al.* 1996). Sex differences in the pattern of GH secretion are not so clear-cut in humans, but there is a noticeably higher serum GH concentration in women than in men (Winer *et al.* 1990, Chapman *et al.* 1994). In addition to being sex-dependent, the pattern of spontaneous GH release is also age-dependent (Eden 1979, Ho *et al.* 1987, Kamegai *et al.* 1999). In all mammalian species studied to date, circulating GH concentrations are elevated during the perinatal period and then decline progressively to the time of weaning (Rieutort 1974, Strosser & Mialhe 1975). A secondary rise in circulating GH concentrations occurs during sexual maturation in both males and females, and at this time the pattern of GH secretion

becomes gender specific as result of hypothalamic function modification by gonadal factors (Strosser & Mialhe 1975, Muller *et al.* 1999). The decrease in GH serum concentration with aging very likely results from decreased pituitary secretion, secondary to enhanced insulin-like growth factor-I (IGF-I) hypothalamo-pituitary feedback, or to changes in the pituitary sensitivity to SS and GHRH (Muller *et al.* 1999). In rats, the decrease in circulating GH concentrations was observed from d1 to d20 and it was associated with an increase in pituitary responsiveness to SS (Oliver *et al.* 1982, Torronteras *et al.* 1997). In addition, juvenile and adult pituitaries are less sensitive to the stimulatory action of GHRH (Szabo *et al.* 1986, Shulman *et al.* 1987), that has been linked to decrease in GHRH receptor synthesis (Korytko *et al.* 1996, Kamegai *et al.* 1999).

Mechanism of GH secretion

Pituitary cells have a regulated pathway utilized by secretory vesicles that fuse with the plasma membrane. Secretory granules of the endocrine cells store and release peptide hormones and are the counterpart of neuronal large dense-core vesicles (Jahn & De Camilli 1991). Translucent vesicles of pituitary cells are designated as synaptic-like microvesicles because of their similar appearance, molecular constituents and exocytotic recycling to synaptic vesicles. Synaptic proteins have been shown to be essential for the life cycle and exocytosis of synaptic vesicles at the nerve terminal. Purification of N-ethylmaleimide-sensitive fusion protein (NSF) and soluble NSF attachment proteins (SNAPs) initiated the search for the mechanism of vesicular transport and release (Sollner *et al.* 1993a,b). An affinity purification procedure based on the natural binding of NSF and SNAPs to their targets was used to isolate SNAP receptors (SNAREs). Recently, synaptic proteins including Rab3a, synaptotagmin, syntaxin 1, SNAP-25, synaptophysin, and synaptobrevin isoforms (synaptobrevin 2 and cellubrevin) were found in pituitary cells (Redecker *et al.* 1995, Jacobsson & Meister 1996, Majo *et al.* 1998). These results suggest similar secretory mechanisms for synaptic vesicles and secretory organelles in both neuronal cells and pituitary cells, including somatotropes.

Regulation of GH secretion

The regulation of GH secretion has been the focus of research since Reichlin in 1960 observed that in rat surgical destruction of the ventromedial hypothalamus slows growth velocity (Reichlin 1960). First, at the end of 1960s and during 1970s GH release inhibiting hormone or somatostatin was discovered (Brazeau *et al.* 1973). About 10 years later GH-releasing factor was identified and sequenced (Zafar *et al.* 1979, Rivier *et al.* 1982). A dual complex interplay between these two hypothalamic factors has a primary role in the regulation of GH secretion from anterior pituitary (AP) gland in mammals. GHRH, stimulates GH synthesis and release, and somatotrope proliferation, while SS, through its inhibitory influences modulates GH release and regulates GH pulsatility (Muller *et al.* 1999). GHRH- and SS-containing neurons, reside primarily in the hypothalamic arcuate nucleus (ARC) and the periventricular nucleus (PVN), respectively (Muller *et al.* 1999). The presence of the SS axons and perikarya also in ARC and ventromedial nucleus (VMN), two areas from which GHRH neurons originate, indicate the existence of an anatomic and functional peptide interaction (Liposits *et al.* 1988). The concept that GHRH and SS interact within CNS to modulate GH secretion is further suggested by colocalization of SS receptors and GHRH producing cells within the ARC (Bertherat *et al.* 1992). According to the general model proposed by Tannenbaum and Ling (1984) GHRH release during troughs in sinusoidal pattern of SS release induces the episodic release of GH, and the rise in SS suppresses baseline release. Evidence has accumulated that this traditional modest model has to be expanded to include other hypothalamic and peripheral factors that can be involved in regulation of GH secretion.

GH feeds back on the hypothalamus to regulate its own secretion. The feedback regulation of GH presumably involves either activation of SS or inhibition of GHRH (Chan *et al.* 1996a). It seems that neuropeptide Y (NPY) and galanin are involved in feedback control of GH (Chan *et al.* 1996a,b). Beside short loop effects of GH on the hypothalamic neurons, long-loop feedback regulation of GH secretion might be operated by IGF-I, nonesterified fatty acids (NEFA) and glucose (Muller *et al.* 1999). In addition, GHRH and SS neurons are subject to modulation by complex network of neurotransmitters, especially noradrenergic and cholinergic, and other hypothalamic peptides, and they are likely the final

mediators of metabolic, endocrine, neural and immune influences for GH secretion (Muller *et al.* 1999; Table 1).

Table 1 Neurotransmitters and growth hormone secretion:

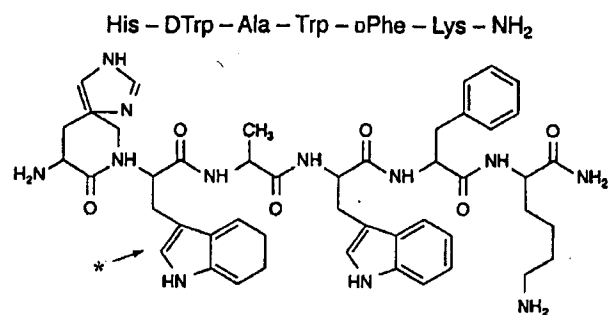
Subjects	Animals	Humans
Epinephrine (E)	↑	↑ ^{a,b}
α_1	↓	↑ ↓ ?
α_2	↑	↑
β_1	↑ ^c	↓
β_2	↓	↓
Norepinephrine (NE)	↑	↑
Dopamine (DA)	↑	↑ ↓
5-hydroxytryptamine (5-HT)	↑ ↓ ^d	↑ ↓ ?
5-HT ₁	↑ ^e	↑ ^f
5-HT ₂	↑	↑
Acetylcholine (ACh)	↑ ^g	↑ ^g
Histamine (H)	↑	—
H ₁	↓	↑
H ₂	↑ ?	↑
γ -Amino butyric acid (GABA)	↑ ↓	↑
Glutamate	↑ ^h	→

↑, Stimulation; ↑, slight stimulation; ↓, inhibition; →, no effect; —, action not ascertained; ?, action still questionable. ^a In combination with propranolol; ^b inhibitory in acromegaly and *in vitro* anterior pituitary; ^c *in vitro*; ^d inhibition in dog; ^e 5-HT_{1C} subtype; ^f 5-HT_{1D} subtype; ^g muscarinic receptors; ^h *N*-methyl-D-aspartate (NMDA) and non-NMDA receptors (modified from Müller *et al.* 1999).

The discovery of family of GH-secretagogues (GHS), which includes GH-releasing peptides (GHRP), and non-peptidergic mimetics, that stimulate GH release both *in vivo* and *in vitro* in several species, suggest that beside GHRH and SS, the third component may play relevant role in GH secretion (Bowers 1996, Smith *et al.* 1996a). Although the exact mechanism of action of GHS has not been fully established, they probably act on both the pituitary and hypothalamus (Dickson *et al.* 1995, Casanueva & Dieguez 1999). It is proposed that exogenous GHS may involve regulatory factors in addition to GHRH and SS, and induce the release of another hypothalamic factor with GH-releasing capabilities designated as U-factor (Bowers *et al.* 1991, Casanueva & Dieguez 1999). Cloning of specific GHS receptors (GHS-R) (Howard *et al.* 1996), and the isolation and characterization of an endogenous ligand for GHS-R, named ghrelin (Kojima *et al.* 1999), implicates GHS as a new physiological system in growth regulation (Tschop *et al.* 2000, Nakazato *et al.* 2001).

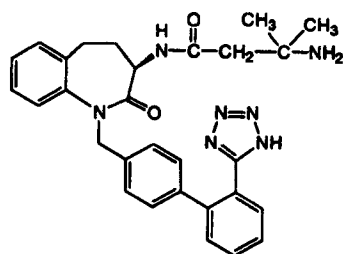
GH- secretagogues and GH- secretagogues receptor

In the late 1970s and early 1980s, the field of GHS research grew out of studies conducted on opioid peptides. After finding that some opioid peptide derivatives had weak GH-releasing activity, in search for more potent GHS Bowers and coworkers tested the GH releasing properties of many peptides, derived from structural modification of Leu- and Met-enkephalin. The data obtained from complex conformational energy calculations were used to relate structural features to the tested GH-releasing capabilities. Subsequent substitutions of amino acids with specific chemical properties at the selected positions of peptide have improved binding and intrinsic activity of these compounds (Bowers *et al.* 1980, 1984, Momany *et al.* 1984). This approach resulted in 1980 with the discovery of the first highly potent GH-releasing hexapeptide named GHRP-6 (His-D-Trp-Ala-Trp-D-Phe-Lys-NH₂; Fig.1) (Bowers *et al.* 1984). GHRP-6 released GH both *in vitro* and *in vivo*, and it was active in several animal species and in humans (Bowers *et al.* 1984, Ilson *et al.* 1989). Since the discovery of GHRP-6 a range of GH-releasing peptides has been developed with varying potency, including hexapeptides hexarelin and GHRP-2, heptapeptide GHRP-1, octapeptide Tyr-Ala-hexarelin as well as penta-, tetra-, pseudotripeptides and some cyclic peptides (Camanni *et al.* 1998, Casanueva & Dieguez 1999).

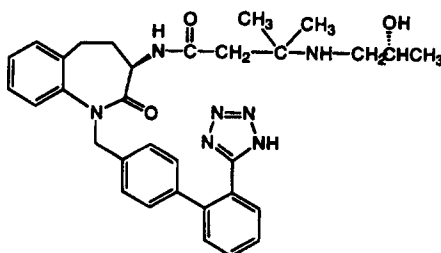


*=(CH₃)-hexarelin

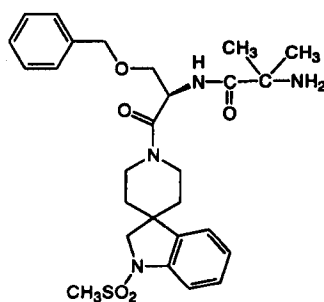
GHRP-6



L-692,429



L-692,585



MK-0677

Figure 1 Structures of peptidyl and nonpeptidyl GH secretagogues (modified from Smith *et al.* 1993 and Casanueva & Dieguez 1999).

Although GHRP were based on an opioid peptide, they were devoid of opioid activity and it was shown that GHRP effectively release GH in animal and man after intravenous, subcutaneous, intranasal, and even oral administration (Camanni *et al.* 1998). Because of the limited oral bioavailability of GHRP, Merck researchers in 1988 embarked on a research program to discover a nonpeptidyl mimic of GHRP-6 with improved oral bioavailability and pharmacokinetics properties (Smith *et al.* 1993, 1996a, 1997). Benzolactam L-692,429 (Fig.1) was the first identified selective nonpeptide GH secretagogue (Smith *et al.* 1993). The *in vivo* specificity of L-692,429 was first evaluated in beagles (Smith *et al.* 1993), and subsequently in human studies (Gertz *et al.* 1993). In common with GHRP-6 (Huhn *et al.* 1993), constant infusion of L-692,429 for 24 h in man provided pulsatile GH release (Chapman *et al.* 1996). Low oral availability of L-692,429 in animal models and modest potency in humans initiated detailed investigation of the structure-activity relationships for benzolactams. The basic amine in the benzolactam class of secretagogues was shown to be critical for GH releasing activity and series of amino substituents were designed to modulate basicity and lipophilicity (Wyvratt 1996). L-692,585 (L-585; Fig. 1), a 2-hydroxypropyl analogue of L-692,429, was found to be approximately 20-fold more potent than L-692,429 in the rat pituitary assays (Schoen *et al.*, 1994), and in beagles *in vivo* (Jacks *et al.* 1994). Infusion of L-585 into guinea pigs was shown to initiate and amplify pulsatile GH release (Fairhall *et al.* 1995). The spiroindoline MK0677 (Fig. 1) was another nonpeptidyl secretagogue evolved through structural modification and sought for orally active molecules more suitable for chronic use. Oral bioavailability of MK0677 has been >60 %, that was much bigger comparing to 2 % and 1% oral bioavailability of L-692,429 and GHRP-6, respectively (Smith *et al.* 1996b). Chronic oral treatment of dogs once daily with MK0677 initiated an amplified pulsatile pattern of GH release, and it was proposed that this class of secretagogue may indeed mimic an endogenous hormone responsible for pulsatile GH release (Smith *et al.* 1996b).

It became clear from early studies that the GHS elicited their effects on GH secretion through receptors and signaling pathways distinct from that of endogenous GHRH (Wu *et al.* 1996). This conclusion was made based on findings that GHRH receptor antagonist inhibited GHRH-stimulated GH release but not GHS-stimulated GH release (Wu *et al.* 1996), and

putative GHS-R antagonist did not affect GH release in response to GHRH (Cheng *et al.* 1989). In addition, GHRH and GHS had synergistic or additive effect on GH release when they were coadministered, and there was no cross-desensitization between GHRH and GHS in terms of GH release, whereas homologue desensitization occurred (Wu *et al.* 1994a,b, Bowers 1996). The synergistic GH response supported an independent and complementary relationship of GHRH and GHS.

The GHS-R was cloned and characterized in several species and it was shown that this receptor mediates the activity of GHRP and nonpeptidyl ligands, (Howard *et al.* 1996, Smith *et al.* 1996b, McKee *et al.* 1997). The identification of the GHS-R confirmed that GHS indeed form part of an alternative physiological system for the regulation of GH secretion, in addition to GHRH and SS. Molecular analysis has shown that cDNA encoding GHS-R is highly conserved among various mammalian species, including human, chimpanzee, swine, bovine, rat and mouse (Howard *et al.* 1996, Mitchell *et al.* 2001). The GHS-R was found to be classical G-protein coupled receptor, containing seven putative alpha-helical membrane spanning segments and three intracellular and extracellular loops. This receptor appears to be the first of a new family of G-protein-coupled receptors, since it does not show significant functional homology with any other proteins and receptor known so far (Howard *et al.* 1996). Its closest relatives are neurotensin and motilin receptors with 35 % and 52 % protein sequence identity (Howard *et al.* 1996, Feighner *et al.* 1999).

To date two different subtypes of GHS-R have been described, types 1a and 1b. In humans and swine the GHS-R subtype 1a (Fig. 2) is a functional, 366 amino acid polypeptide with seven transmembrane (TM) domains (Howard *et al.* 1996). The rat GHS-R type 1a is also 7 TM receptor, but compared with the human and swine GHS-R is two amino acids shorter, with a loss of one residue each in the amino-terminal extracellular domain and the third intracellular loop (McKee *et al.* 1997). The subtype 1b is a truncated version of GHS-R with only 5 TM domains and with no measurable functional activity in cell-based assays (Howard *et al.* 1996, McKee *et al.* 1997).

The GHS-R are mainly present at the pituitary and hypothalamic level but also in other areas of the central nervous system (CNS), such as the cerebral cortex, hippocampus, medulla oblongata and choroid plexus (Howard *et al.* 1996, Muccioli *et al.* 1998). The

existence of GHS-R at the pituitary level and within the CNS explains the neuroendocrine and extraneuroendocrine activities of GHS, such as the control of sleep and food intake (Frieboes *et al.* 1995, Locke *et al.* 1995). GHS-R are also present at the peripheral level in both endocrine and nonendocrine tissues (Papotti *et al.* 2000), but their functional significance is still unknown. Recent findings suggest that at least in cardiovascular system and thyroid gland GHS-R could mediate GH-independent GHS activities (Ghigo *et al.* 2001).

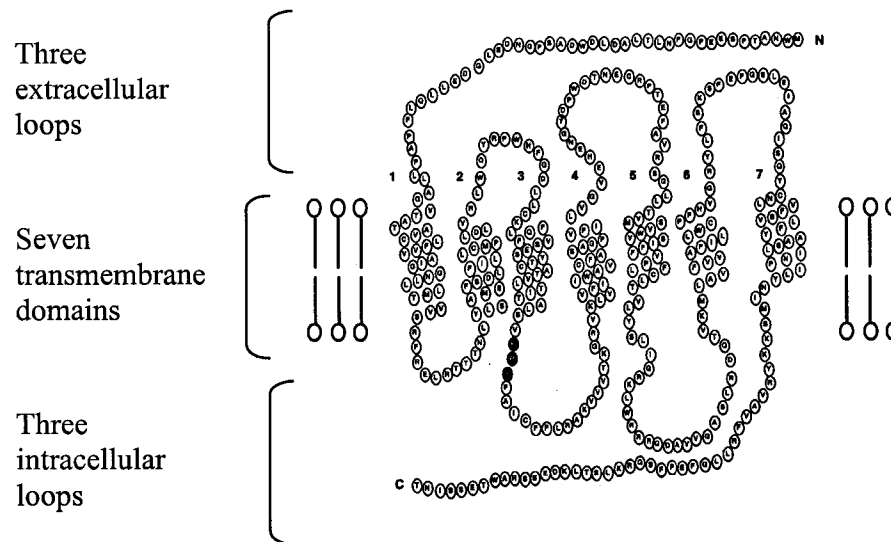


Figure 2 Predicted membrane topology and amino acid sequence of the human type 1a GHS-R (modified from Smith *et al.* 1997).

All peptide and non-peptide ligand classes share a common binding domain in TM-3 region of GHS-R, while contacts in other TM domains and extracellular loop 1 of the receptor reveal specificity for the different GHS (Feighner *et al.* 1998). Changes in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), cyclic adenosine monophosphate (cAMP), protein kinase A (PKA) and C (PKC), and phospholipase C (PLC) are possible signaling systems involved in the action of GHS (Smith *et al.* 1997, Muller *et al.* 1999, Chen 2000). The different signalling systems employed by different GHS in different animal species may reflect existence of more than one subtype of GHS-R (Chen 2000).

Mechanism of action of GHS still remains obscure. Although GHS selectively release GH by a direct pituitary action (Bowers *et al.* 1984, Cheng *et al.* 1989, Sánchez-Hormigo *et al.* 1998), there is good evidence that the GHS also act in the hypothalamus (Dickson *et al.* 1993, Guillaume *et al.* 1994). Moreover, GHS require the presence of a functional hypothalamus to be fully operative (Pombo *et al.* 1995, Popovic *et al.* 1995). The exact pathways through which GHS act in the hypothalamus to release GH are not known. In the hypothalamus GHS-R 1a mRNA is expressed in neuropeptide Y (NPY)/agouti-related protein (AGRP), SS, GHRH and proopiomelanocortin (POMC) neurons (Tannenbaum *et al.* 1998, Willeesen *et al.* 1999). It has been reported that GHS induce *c-fos* expression, a marker of neuronal activity, mainly in NPY and GHRH neurons, but also in POMC and SS neurons (Kamegai *et al.* 1996, Dickson & Luckman 1997). Activation of GHRH neurons by GHS can be direct or indirect through activation of SS and/or NPY neurons (Bluet-Pajot *et al.* 2001). GHS do not appear to inhibit hypothalamic SS release (Guillaume *et al.* 1994, Korbonits & Grossman 1995, Fletcher *et al.* 1996). The issue of whether or not GHS act directly on GHRH neurons is still controversial. The increase in GHRH has been measured in hypothalamic-pituitary portal vessels of sheep after systemic treatment with GHRP (Guillaume *et al.* 1994), but GHRP did not stimulate GHRH release with perfusion of rat hypothalamus *in vitro* (Korbonits & Grossman 1995). It seems that GHS-elicited GH secretion is not mediated by changes in endogenous GHRH or SS, and it is proposed that GHS may induce the release of another hypothalamic factor with GH-releasing capabilities – “U-factor” (Casanueva & Diguez 1999).

Ghrelin

The search for GHS-R endogenous ligand was actively undertaken in the last few years. In 1999, endogenous ligand for GHS-R was discovered by a Japanese research group (Kojima *et al.* 1999). The natural, endogenous ligand for GHS-R was always believed to be another hypothalamic peptide, but surprisingly it was isolated from both the rat and the human stomach. The Japanese research group used a Chinese hamster ovary (CHO) cell line that express GHS-R to monitor changes in $[Ca^{2+}]_i$ and by screening extracts from several tissues, it was observed that the highest level of GHS-R activation occurs in response to

stomach extract. The purified ligand was named ghrelin. “Ghre” is the Proto-Indo-European root for the word growth and the suffix “relin” signifies that this peptide stimulates GH release (Kojima *et al.* 1999).

Ghrelin is relatively charged linear, non-C-terminal amidated 28 amino acids peptide (Fig. 3) with a significant number of functional groups. The third serine residue in ghrelin is *n*-octanoylated, and this posttranslation modification by an acyl acid is unique among mammalian bioactive peptides. The *n*-octanoyl group adds hydrophobic property to the N-terminus that may facilitate entry and distribution in the brain. Without octanoylation, ghrelin is biologically inactive (Kojima *et al.* 1999, Bednarek *et al.* 2000). Interestingly, it was shown that the first four to five residues of ghrelin activate GHS-R as efficiently as a full length of ghrelin (Bednarek *et al.* 2000). The cDNAs for rat, mouse, dog and human ghrelin have been cloned (Kojima *et al.* 1999, Tanaka *et al.* 2001, Tomasetto *et al.* 2001). The structure of ghrelin is highly preserved among different species, and human ghrelin differs from rat ghrelin in two residues (Kojima *et al.* 1999). Sequence comparison indicated that ghrelin and motilin share partial homology. Human ghrelin and human motilin have 36% identity, and GHS-R show a remarkable overlap with motilin receptor (Asakawa *et al.* 2001). These facts suggest that ghrelin and motilin might have evolved from a common ancestral peptide.

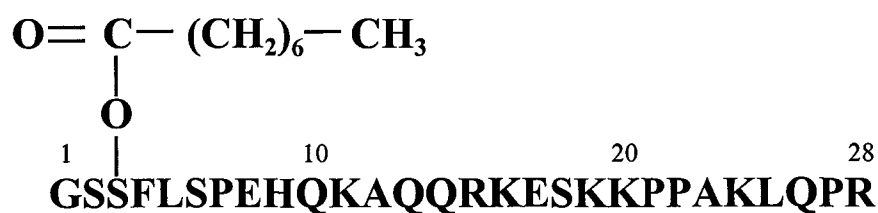


Figure 3 Structure of rat ghrelin (modified from Kojima *et al.* 1999).

Following the discovery of ghrelin, a second endogenous ligand for GHS-R was also isolated from the rat stomach (Hosoda *et al.* 2000). This peptide was identical to ghrelin, except for the deletion of Gln 14, and it was called des-Gln 14 ghrelin. Des-Gln 14 ghrelin is encoded by a mRNA created by alternative splicing of the ghrelin gene. The activity of both ghrelins is the same. However, des-Gln 14 ghrelin is only present in low amounts in the stomach, indicating that ghrelin is the major active form (Kojima *et al.* 2001).

Ghrelin mRNA, as was shown by northern blot analysis, is primarily expressed in the mucosal layer of the stomach with concentrations decreasing distally along the intestine (Kojima *et al.* 1999, Date *et al.* 2000). Date *et al.* (2000) demonstrated that stomach ghrelin is present in distinct cell type, X/A like endocrine cells of submucosal layer, or now designated as ghrelin cells. These cells contain round, compact electron-dense granules and are filled with ghrelin. The ghrelin cells are not in continuity with the stomach lumen, but rather are closely associated with the capillary network of the *lamina propria*, suggesting that ghrelin is secreted into the blood vessels (Kojima *et al.* 2001).

Ghrelin expression is not restricted to the gastrointestinal tract, but it is also produced in the pancreas, kidney liver, immune cells, pituitary and hypothalamus (Kojima *et al.* 1999, Mori *et al.* 2000, Hattori *et al.* 2001, Korbonits *et al.* 2001). Ghrelin was also found in rat and human placenta (Gualillo *et al.* 2001), suggesting that ghrelin-GH axis might be active early in development.

Ghrelin receptors (GHS-R) also have widespread distribution in the peripheral tissues. The presence of these receptors was documented in the pituitary, hypothalamus, stomach, heart, blood vessels, lung, pancreas, intestine, kidney, adipose tissue, immune system and in human breast carcinomas (Wang *et al.* 2002). The expression of ghrelin and its receptors in these various tissues suggests multiple paracrine, autocrine and endocrine, physiological and pathophysiological roles for ghrelin.

Based on the evidence that nonnatural GHS are the strong activators of the GH release, it was reasonable to expect that ghrelin, as an endogenous ligand for GHS-R, possesses GH – releasing activity. In fact, it has been shown that ghrelin acts directly on the somatotropes, and in a dose dependent manner stimulates GH secretion from rat pituitary cells in culture. This stimulatory effect of ghrelin on GH secretion is specific, and does not affect other pituitary hormones, even at high ghrelin doses (Kojima *et al.* 1999). Furthermore, it was found that in rodents ghrelin, given systemically or into the brain (*icv*), stimulates GH secretion (Kojima *et al.* 1999, Date *et al.* 2000, Tolle *et al.* 2001). After intravenous injection of ghrelin to rat, plasma GH levels peaked 5-10 min after injection, and returned to basal levels 1h later (Tolle *et al.* 2001). It has recently been shown that ghrelin in a dose dependent manner stimulates GH-release in humans (Takaya *et al.* 2000, Arvat *et al.* 2001, Hataya *et al.*

2001). In normal male adults, *iv* administration of ghrelin in doses of 0.2, 1 and 5 $\mu\text{g/kg}$ induced prompt increase in circulating GH levels that reached peak values 30 min after injection. Adrenocorticotropin (ACTH), cortisol and prolactin (PRL) levels were also elevated after ghrelin injection. At the low *iv* bolus dosage ghrelin induced only minimum peak values of these hormones, suggesting that at physiological concentrations, ghrelin possibly would not increase ACTH or PRL (Takaya *et al.* 2000, Arvat *et al.* 2001). Ghrelin exhibited a similar time course for GH release to those of GHS (Takaya *et al.* 2000). The potency of ghrelin for GH release was similar to those of GHS, but much greater than GHRH, indicating a distinctive pharmacological action (Takaya *et al.* 2000, Arvat *et al.* 2001). At small dose ghrelin synergised with GHRH on GH secretion, that also suggested different sites of action for these hormones (Arvat *et al.* 2001, Hataya *et al.* 2001). On the other hand, several studies have shown commonality of action of GHRP and ghrelin. The endocrine responses to ghrelin were not modified by the coadministration of hexarelin, a nonnatural peptidyl GHS (Arvat *et al.* 2001). In experiments with repeated administration of ghrelin or GHRP-2, desensitization of the GH response was induced homologously and also heterologously in crossover studies. Also, combined ghrelin and GHRP-2 at maximal dosages induced the same magnitude of GH release as when the peptides were administered alone (Bowers 2001). Together, these *in vivo* assays confirmed that ghrelin is a new hormone playing an important role in GH secretion. Still is unknown whether ghrelin itself stimulates pituitary GH synthesis either directly or indirectly via the release and/or augmentation of the pituitary action of GHRH and other hypothalamic factors.

A large number of animal and human studies have shown that in addition to role in regulation of GH secretion, ghrelin is involved in the regulation of energy balance. The positive energy balance induced by ghrelin seems to be necessary to maximize the anabolic actions of GH (Inui 2001). Ghrelin has a complex function in growth regulation by both stimulating GH secretion, and by stimulating feeding. Adipogenic as well as orexigenic effects of ghrelin are independent from its ability to stimulate GH secretion. It was shown that ghrelin increases feeding in rats that are genetically deficient in GH (Nakazato *et al.* 2001).

In rodents, ghrelin can stimulate food intake when given either systemically or *icv* (Asakawa *et al.* 2001, Nakazato *et al.* 2001). The central injection of ghrelin produced more robust and sustained food intake, indicating a central mode of action (Tschop *et al.* 2000). Fasting and hypoglycemia increased ghrelin levels, whereas intake of food, especially carbohydrates, decreased ghrelin secretion (Tschop *et al.* 2000, Asakawa *et al.* 2001, Nakazato *et al.* 2001).

The orexigenic effect of ghrelin was also observed in humans (Arvat *et al.* 2001). In humans, circulating ghrelin levels were decreased in obesity (Tschop *et al.*, 2001*b*) and acute feeding states (Tschop *et al.* 2001*a*), whereas plasma levels of ghrelin were increased by fasting (Cummings *et al.* 2001). Recently, pre-meal rise of human ghrelin was observed, suggesting a possible role of ghrelin as hunger signal triggering meal initiation (Cummings *et al.* 2001).

The orexigenic activity of ghrelin is most likely mediated by its action on the arcuate nucleus of the hypothalamus. The arcuate nucleus is a major hypothalamic site for regulation of feeding behaviour and body weight since it harbors NPY, agouti-related peptide (AGRP), cocaine and amphetamine-regulated transcript (CART), and pro-opiomelanocortin (POMC) (Woods *et al.* 1998, Abbott *et al.* 2001). NPY and AGRP are orexigenic, whereas CART and POMC are anorexic peptides. GHS-R mRNA has been found in the hypothalamus, and immunohistochemical analyses indicated that ghrelin-containing cells are localized in the arcuate nucleus (Kojima *et al.* 1999). It is also possible that the fatty acid moiety facilitates passage across the blood-brain barrier, resulting in hypothalamic actions of systemic ghrelin (Inui 2001). After intracerebroventricular administration of ghrelin, Fos protein, a marker of neuronal activation, was robust in regions involved in regulation of feeding, including NPY and AGRP neurons (Kojima *et al.* 2001, Nakazato *et al.* 2001). It was also shown that Y1-receptor antagonists as well as melanocortin agonist and antisera to both NPY and AGRP might interfere with ghrelin's feeding-inducing effect (Asakawa *et al.* 2001, Nakazato *et al.* 2001). However, the effect of ghrelin on feeding and adiposity was not diminished in NPY lacking mice, indicating a crucial role of AGRP in the mediation of ghrelin's effects on energy balance (Tschop *et al.* 2002). The effect of ghrelin on metabolism seems to be exact opposite to the leptin. Leptin is an adipocyte-derived blood-born satiety factor that acts

directly on the hypothalamus, where it regulates a large number of molecules implicated in energy homeostasis. Administration of leptin either centrally or peripherally inhibits appetite and adiposity and augments energy expenditure (Friedman & Halaas 1998). Seemingly, these opposing effects of leptin and ghrelin on appetite are mediated by NPY, AGRP and γ -aminobutyric acid (Horvath *et al.* 2001, Nakazato *et al.* 2001, Shintani *et al.* 2001). The exact mechanism of the interaction between leptin and ghrelin remain to be elucidated in the future studies. It is important to clarify the role of gastric ghrelin and the NPY/AGRP pathway in the pathogenesis of diet-induced obesity, because obesity and related disorders are among the leading causes of illness and mortality in the developed world (Mokdad *et al.* 2000).

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CHAPTER 2. MECHANISM OF ACTION OF GROWTH HORMONE SECRETAGOGUE, L-692,585, ON ISOLATED PORCINE SOMATOTROPHS

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Abstract

The effects of a growth hormone (GH)-secretagogue, L-692,585 (L-585), and human growth hormone releasing hormone (hGHRH) on calcium transient and GH release were investigated in isolated porcine pituitary cells using calcium imaging and the reverse hemolytic plaque assay (RHPA). Somatotrophs were functionally identified by application of hGHRH. All cells that responded to hGHRH responded to L-585 application. Perfusion application of 10 μ M hGHRH and L-585 for 2 min resulted in an increase in $[Ca^{2+}]_i$ of 53 ± 1 nM (mean \pm SEM) ($P < 0.01$) and 68 ± 2 nM ($P < 0.01$), respectively. The L-585 response was characterized by an initial increase in $[Ca^{2+}]_i$ followed by decline to a plateau level above the baseline. Concurrent calcium imaging with RHPA indicated that the L-585-evoked increase in $[Ca^{2+}]_i$ coincided with GH release. L-585 significantly increased the percentage of plaque forming cells (24 ± 3 vs 40 ± 6 %; $P < 0.05$) and mean area of plaques ($1,892 \pm 177$ vs $3,641 \pm 189$ μ m²; $P < 0.01$) indicating increased GH release. Substance P-analog ([D-Arg¹, D-Phe⁵, D-Trp^{7,11}]-SP) blocked, and the hGHRH receptor antagonist [(Phenylac-Tyr¹, D-Arg², p-chloro-Phe⁶, Homoarg⁹, Tyr (Me)¹⁰, Abu¹⁵, Nle²⁷, D-Arg²⁸, Homoarg²⁹)-GRF (1-29) amide] decreased the stimulatory effect of hGHRH. These failed to block the stimulatory effect of L-585 suggesting different receptor for L-585 from GHRH receptor. The hGHRH-induced calcium transients and initial peak increase induced by L-585 were significantly decreased by removal of calcium from bathing medium or the addition of nifedipine, a L-calcium channel

blocker. The plateau component of L-585-induced calcium change was abolished by removal of calcium and nifedipine. These results suggest an involvement of calcium channels in GH release. Either SQ-22536, an adenylyl cyclase (AC) inhibitor, or U73122, a phospholipase C (PLC) inhibitor, blocked the stimulatory effects of hGHRH and L-585 on $[Ca^{2+}]_i$ transient indicating the involvement of AC-cAMP and PLC- IP_3 pathways. These results further suggest calcium mobilization from internal stores during the first phase of the L-585 induced increase in $[Ca^{2+}]_i$ whereas calcium influx during the second phase is a consequence of somatotrope depolarization.

Introduction

GROWTH HORMONE (GH) secretion is controlled by hypothalamic stimulatory- and inhibitory-releasing hormones, GH-releasing hormone (GHRH) and GH release-inhibiting hormone (GHRH) or somatostatin (SS). GH also participates in its own rhythmic secretion through feedback action on GHRH and SS neurons (Chan *et al.* 1996). Pulsatile GH secretion can be stimulated and amplified by novel compounds known as growth hormone secretagogues (GHS) (Casanueva & Dieguez 1999). The mechanism of action of GHS is not fully established. GHS-elicited GH secretion involves both a direct effect and an indirect effect on the hypothalamus, suggesting that exogenous GHS may induce the release of another hypothalamic factor with GH-releasing capabilities (U-factor) (Bowers *et al.* 1991, Casanueva & Dieguez 1999). Cloning of the human GHS receptor (Howard *et al.* 1996) and the isolation and characterization of an endogenous GHS, designated as ghrelin (Kojima *et al.* 1999), implicates GHS as a new physiological system in growth regulation by stimulating feeding and release of GH (Nakazato *et al.* 2001).

The growth hormone releasing peptides (GHRPs) were the first identified compounds in the class of GHS. Bowers and coworkers reported the discovery of a series of peptides derived from Leu- and Met-enkephalins that specifically released GH from the pituitary gland of animals and humans (Bowers *et al.* 1991). The biological action of GHRPs was considered different from GHRH. Benzolactam and spiroindolamine GHS have been developed with improved oral bioavailability and pharmacokinetic properties (Smith *et al.* 1993, 1997); L-692,429 being the first described nonpeptidyl GHS (Smith *et al.* 1993). L-

692,585 (L-585) is a nonpeptidyl GHS 10- to 20-fold more potent than L-692,429, as based on *in vitro* and *in vivo* studies and with no detectable change in receptor affinity (Jacks *et al.* 1994, Schoen *et al.* 1994). The GHS activity of L-585 has been reported in several species including beagles (Jacks *et al.* 1994), sheep (Guillaume *et al.* 1994) and swine (Hickey *et al.* 1996). *In vivo* experiments have shown that GHS in combination with GHRH augments GH release, and it is suggested that L-585 acts directly on somatotropes causing GH release (Smith *et al.* 1993). Besides direct effects on somatotropes, L-585 exerts central effects and requires an intact hypothalamic-pituitary axis for optimal GH release (Fairhall *et al.* 1996, Hickey *et al.* 1996). Experiments in the pig *in vivo* have shown that L-585 also stimulates GH secretion by acting in combination with GHRH to interrupt the inhibitory tone of somatostatin (Hickey *et al.* 1996).

The present studies examine the signal transduction mechanism of GHS, L-585 on calcium transient and GH release from isolated porcine somatotropes. An understanding of the molecular mechanisms by which GHS modulate GH secretion is of particular interest because of their biochemical simplicity and efficacy of oral administration.

Materials and Methods

Chemicals

Fetal bovine serum (FBS), horse serum (HS), minimum essential medium (MEM), Dulbecco's modified Eagle's medium (DMEM), Earle's balanced salt solutions (EBSS) and guinea pig complement were purchased from Gibco-Invitrogen Co. (Carlsbad, CA, USA). HEPES, ethyleneglycol-bis-(β -aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), papain solution, trypsin-inhibitor, penicillin-streptomycin solution, poly-L-lysine, L-cysteine, L-glutamine, sodium pyruvate, bovine serum albumin (BSA), nifedipine, and *Staphylococcal* protein-A were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Ovine red blood cells (oRBC) were purchased from Colorado Serum (Denver, CO, USA). Glucose, sodium bicarbonate (NaHCO_3), paraformaldehyde, and chromium chloride were obtained from Fisher Chemical (Fair Lawn, NJ, USA). Fura 2/AM and 4-bromo-A23187 were purchased from Molecular Probes (Eugene, OR, USA). ABC kit was obtained from Vector Laboratories (Burlingame, CA, USA). U73122 and SQ-22536 were purchased from Biomol Research

Laboratories (Plymouth Meeting, PA, USA). Somatostatin (SS), Substance P analog, (D-Arg¹, D-Phe⁵, D-Trp^{7,11})-SP, and GHRH antagonist, (Phenylac-Tyr¹, D-Arg², p-chloro-Phe⁶, Homoarg⁹, Tyr (Me)¹⁰, Abu¹⁵, Nle²⁷, D-Arg²⁸, Homoarg²⁹)-GRF (1-29) amide (human), were obtained from Bachem California, Inc. (Torrance, CA, USA). hGHRH and anti-porcine GH antibody were gifts from Dr A F Parlow, NIDDK National Hormone and Pituitary Program (Torrance, CA, USA). L-692,585 (L-585) was a gift from Dr G J Hickey, Merck Research Laboratories, Rahway, NJ, USA.

Experimental animals

Yorkshire pigs, raised at the Iowa State University Animal Nutrition Farm, were used for these experiments. Animal care and experimental protocols were in accordance with the guidelines and approval of the Iowa State University Committee on Animal Care.

Preparation of cell cultures

Newborn pigs, 1-8 days of age, were killed with electrical shock and decapitated. Pituitary glands were immediately removed. Total number of animals used was 32. The pituitary glands were collected in cold sterile EBSS solution (4 °C). Anterior lobes were transferred to a sterile cold (4 °C) MEM-0.1% BSA medium. Primary cell cultures from neonatal anterior pituitary gland were established using a modified method of Huettnner & Baughman (1986). Tissues from two animals were incubated 50 min at 37 °C in 2 ml of EBSS-papain solution (1.54 mg/ml). After incubation, tissue was rinsed with EBSS solution and incubated for 5 min in trypsin-inhibitor solution (1 mg/ml). After being rinsed, once with EBSS solution and once with DMEM-0.1% BSA medium, the tissue was mechanically dispersed in DMEM-0.1% BSA medium by triturating through a firepolished glass pipette. The undigested tissue was allowed to sediment. The supernatant containing cells was removed and filtered through a sterile filter. Cells were plated onto poly-L-lysine (0.1 mg/ml; 100,000 kD) coated glass coverslips (at a density of 2×10^5 cells). Cells were allowed to attach to coverslips, and then after 3-4 h DMEM-0.1% BSA medium was exchanged with DMEM medium supplemented with 10% horse serum and 1 ml penicillin-streptomycin solution per 100 ml medium. Cultures were maintained at 37 °C in a humidified 5% CO₂/95% air atmosphere. Experiments

were carried out after 2 days in culture. The presence of somatotropes was confirmed by immunocytochemical methods.

Immunocytochemistry staining

After fixation with 4% paraformaldehyde for 30 min at room temperature, cells were incubated for 30 min in a 50% goat serum solution containing 1% BSA and 100 mM L-lysine to block nonspecific binding and 0.4 % Triton X-100 to permeabilize the membrane. To stain the somatotropes, cultures of anterior pituitary gland were incubated in polyclonal anti-porcine GH antibody (dilution 1:50,000). Antibody visualization was accomplished by using a Vectastain ABC kit (Vector) and the nickel-enhanced 3-3' diaminobenzidine method (Jeftinija *et al.* 1992).

Intracellular calcium imaging

The effect of secretagogues on intracellular calcium concentration $[Ca^{+2}]_i$ was evaluated by ratiometric imaging techniques (Parpura *et al.* 1994). Cells were loaded with Fura 2-AM for 40-60 min at 37 °C. 1 µl of 25% (w/w) of Pluronic F-127 was mixed with 4 nM of AM ester to aid solubilization of the ester into aqueous medium. Coverslips containing pituitary cells were washed with HEPES-buffered solution and further incubated for 10 min at 37 °C to allow deesterification of Fura 2-AM. All image processing and analysis were performed using an Attoflour system with Zeiss microscope. Background subtraction and ratio images were used to calculate the $[Ca^{+2}]_i$ according to Equation 5 of Grynkiewicz *et al.* (1985). Using wavelengths of 340 and 380 nm, Fura 2-AM was excited and the emitted light was collected at 520 nm.

Reverse hemolytic plaque assay (RHPA) for measurement of GH release in culture medium

The RHPA was established according to the method of Taylor and Clark (Taylor & Clark 1994). *Staphylococcus* protein A was coupled to ovine red blood cells using 0.1% $CrCl_3$. After dissociation, pituitary cells were incubated for 48 h to allow them to recover biological responsiveness. After incubation, the cells were briefly exposed to trypsin (2.5 mg trypsin in 10 ml MEM-0.1% BSA) to detach them from the culture dish. Following gentle triturating by

a firepolished glass pipette, the cells were separated by centrifugation ($1,500 \times g$ for 10 min), washed twice in MEM-0.1% BSA, and resuspended in DMEM-0.1% BSA. The working cell dilution was 2.5×10^5 cells/ml. Equal amounts of the single secretory cell suspension and a 50% protein-A-labeled erythrocyte solution were mixed. The mixture was infused into Cunningham slide chambers and incubated at 37 °C in 95% air-5% CO₂ for 50 min. Pretreatment of the glass surface with poly-L-lysine (0.1 mg/ml; 100,000 kD) ensured anchorage of the cells. After incubation the chambers were rinsed with DMEM-0.1 % BSA. The monkey anti-porcine GH serum, diluted 1:60 in DMEM-0.1 % BSA, was then infused into the chambers alone or with L-585 (100 nM). The optimal incubation period was established by a preliminary study in which the maximum percentage of plaque-forming GH cells was achieved at 3 h. Plaque formation was initiated by infusion of guinea pig complement in a final dilution of 1:40 in DMEM-0.1 % BSA, and the reaction was terminated after 50 min by the infusion of 1% solution of glutaraldehyde. Omission of any of the assay reagents, i.e., antiserum, complement, protein-A, resulted in failure to form hemolytic plaque.

In every experiment, each treatment (with secretagogue or vehicle) was run in triplicate. Three separate experiments were performed. On each slide, the percentage of plaque forming cells (% PFC) was determined by counting the number of plaques among 200 pituitary cells. The mean plaque area (MPA) of 100 plaques/slide was measured by using a calibrated ocular reticule. The total secretion index, representing integrated hormone secretion per 100 pituitary cells, was calculated by multiplying the % PFC and MPA (Goth *et al.* 1992; Mitani *et al.* 1996).

Combination of RHPA and measurement of $[Ca^{2+}]_i$

A 1:1 mixture of protein-A-conjugated ovine red blood cells and pituitary cells was incubated for 1 h in Cunningham slide chambers, and then loaded with Fura 2-AM for 40 min at 37 °C. Fura 2-AM was washed from the chamber with DMEM-0.1% BSA. Twenty minutes later the presence of pituitary cells was confirmed under the fluorescent microscope. Calcium measurements were performed using consecutive excitation at 340 and 380 nm. After recording a baseline of $[Ca^{2+}]_i$ for 60 s, anti-porcine GH antibody and secretagogue (10 μ M

L-585) were both introduced into the chamber. Changes in $[Ca^{2+}]_i$ were monitored for 15 min. After recording, cells in the chambers remained on the microscope for 3 h. Complement was introduced into the chamber, leading to lysis of red blood cells adjacent to GH secreting pituitary cells. These plaques retrospectively identified the somatotropes.

Statistical analysis

Independent data are expressed as mean \pm S.E.M. Simple comparisons between two groups were made by Student's *t* test. Multiple comparisons were carried out by one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison Test. Differences were considered as statistically significant at $P < 0.05$.

Results

Morphological identification of somatotropes in culture

Somatotropes in pituitary cell culture were confirmed by immunocytochemical staining. GH immunoreactive cells comprised 40% of the total pituitary cells in cultures. Somatotropes were approximately 10 μ m in diameter (Fig. 1A).

Functional identification of somatotropes in culture

Changes in the intracellular calcium concentration ($\Delta[Ca^{2+}]_i$) mediate a variety of biological responses in both excitable and non excitable cells. GH cells were functionally identified by $[Ca^{2+}]_i$ increase above the baseline following hGHRH application. Perfusion with hGHRH (10 μ M) for 2 min significantly increased $[Ca^{2+}]_i$ by 53 ± 1 nM (mean \pm SEM; $n = 578$; $P < 0.01$; Fig. 1B). Of the cells that responded to hGHRH, all (100%) also responded to L-585, that was applied 10 min after the application of hGHRH. Perfusion with 10 μ M L-585 for 2 min produced a prompt transient increase in $[Ca^{2+}]_i$ followed by the sustained decline to a plateau above the basal level. The initial increase in intracellular calcium reached a peak of 68 ± 2 nM ($n = 578$; $P < 0.01$) approximately 80 s after the onset of L-585 application (Fig. 1B). Potassium at a concentration of 50 mM, a nonselective stimulus, elevated $[Ca^{2+}]_i$ by 53 ± 1 nM ($P < 0.01$) in all cells that responded to hGHRH and L-585 (Fig. 1B). Some cells only

responded to the (nonspecific) stimulation with 50 mM K⁺ whereas others failed to respond to any stimuli (Fig. 1B).

L-585 induced release of GH in a dose dependent manner

To demonstrate that calcium transient in cultured pituitary cells after application of L-585 coincided with GH secretion, we performed experiments where reverse hemolytic plaque assay (RHPA) and [Ca²⁺]_i measurements were done concurrently. Hemolytic plaques were formed only around cells that on calcium imaging responded to L-585, which identified them as GH-secreting somatotropes (Fig. 1C).

Before quantitatively studying the effects of L-585 on GH release, preliminary experiments were performed in which the maximum effective dose (ED_{max}) of L-585 for GH secretion was determined. When incubated with 10 nM, 100 nM, and 10 μM L-585, cultured pig somatotropes dose-dependently released GH with the ED_{max} of 100 nM. Using RHPA, the percentage of plaque forming cells (% PFC), and their mean plaque area (MPA) after 3 h incubation, were quantified. A total secretion index (TSI) was calculated by multiplying % PFC and MPA. Under basal conditions, GH plaques were formed in 24 ± 3 % of the pituitary cells in culture obtained from newborn pigs. The similar % PFC (about 30%) under basal condition was observed in experiments with rats somatotropes (Niimi *et al.* 1994; Mitani *et al.* 1996). The average size of the GH plaques in control cultures treated with vehicle was 1,892 ± 177 μm². Incubation with 100 nM L-585 significantly increased the number and size of plaques. The fraction of somatotropes forming plaques was increased to 40 ± 6 % (*P* < 0.05; Fig. 2A) and the mean plaque area was 1.92 fold greater than controls (3,641 ± 189 μm²; *P* < 0.01; Fig. 2B). L-585 also caused significant increase in TSI (× 10⁻²) (vehicle 5 ± 1 vs 13 ± 2; *P* < 0.01; Fig. 2C).

Stimulatory effect of hGHRH and L-585 is receptor mediated

To study the nature of GHS effect on somatotropes, ligands were applied in succession. The second application of 10 μM hGHRH caused an increase [Ca²⁺]_i in 63% of the cells (60 of 96) that responded to the first application of hGHRH, and 59% of cells (57 of 96) that responded to the first application of 10 μM L-585 responded to repeated application of L-

585. A second application of 10 μ M hGHRH and 10 μ M L-585 did not have an additive effect on the increase in $[Ca^{2+}]_i$ (32 ± 2 vs 20 ± 1 nM; 58 ± 4 vs 25 ± 3 nM; Fig. 3B). Similarly, simultaneous application of hGHRH and L-585 in a concentration of 10 μ M, also did not have an additive effect on the increase of $[Ca^{2+}]_i$ (57 ± 4 nM; $n = 77$; Fig. 3C) but a sustained plateau phase of calcium increase was prolonged compared with either agonist alone. In parallel run controls average increase in $[Ca^{2+}]_i$ after application of 10 μ M GHRH and 10 μ M L-585 alone was 56 ± 3 nM and 66 ± 5 nM, respectively ($n = 74$; Fig 3A).

To determine whether the effects of hGHRH and L-585 were receptor mediated we utilized a GHRH-receptor antagonist and a substance P analog with GHRH antagonistic properties. Application of 10 μ M GHRH-receptor antagonist, (Phenylac-Tyr¹, D-Arg², p-chloro-Phe⁶, Homoarg⁹, Tyr (Me)¹⁰, Abu¹⁵, Nle²⁷, D-Arg²⁸, Homoarg²⁹)-GRF (1-29) amide (human), for 10 min significantly decreased the rise in $[Ca^{2+}]_i$ evoked by hGHRH compared to controls run in parallel (68 ± 8 nM, $n = 27$ vs 47 ± 3 nM, $n = 68$; $P < 0.01$; Fig. 4A,B). In contrast, application of GHRH-receptor antagonist did not influence the stimulatory effect of L-585 (80 ± 11 nM, $n = 27$ vs 80 ± 8 nM, $n = 70$; Fig. 4B). Substance P analog, ([D-Arg¹, D-Phe⁵, D-Trp^{7,11}]-SP), at a concentration of 10 μ M, blocked the stimulatory effect of hGHRH (53 ± 2 nM, $n = 48$ vs 0 nM, $n = 46$) but it did not influence the stimulatory effect of L-585 (75 ± 4 nM, $n = 48$ vs 71 ± 4 nM, $n = 44$; Fig. 4C). These findings demonstrated that hGHRH and L-585 increase intracellular calcium concentration by acting through different receptors in cultured porcine somatotropes.

Somatostatin (SS) dose-dependently decreased stimulatory effect of hGHRH and L-585

Applications of SS at concentrations of 5 and 10 μ M decreased intracellular calcium levels by 15 ± 2 and 26 ± 3 nM, respectively (Fig. 4D). The blocking effect of SS on hGHRH and L-585 evoked increase in $[Ca^{2+}]_i$ was dose dependent. Perfusion application of 10 μ M SS for 5 min before the application of 10 μ M hGHRH and 10 μ M L-585 abolished the stimulatory effect of both agonists (Fig. 4D). After pretreatment of the cultures with 5 μ M SS for 5 min, 59% of cells (26 of 44) responded to 10 μ M hGHRH, and 57% of cells (25 of 44) responded to 10 μ M L-585, while in parallel run controls 94% of cells (47 of 50) responded to

application of agonists. In the presence of 5 μM SS the increase in $[\text{Ca}^{2+}]_i$ evoked by hGHRH was significantly smaller compared with controls (44 ± 3 vs 25 ± 4 nM; $P < 0.01$), while the peak increase of $[\text{Ca}^{2+}]_i$ produced by L-585 was not significantly decreased (70 ± 6 vs 59 ± 6 nM).

Role of membrane depolarization and calcium channels in hGHRH and L-585 stimulatory effect

To investigate the role of extracellular calcium in hGHRH and L-585 induced calcium transient in cultured somatotropes, cells were bathed in calcium depleted HEPES with the addition of 1 mM EGTA to yield an estimated free extracellular calcium level of 26 nM. We found that it was necessary to have some calcium in the bathing medium, otherwise cells detached from the culture substrate. Consistent with the ability of ligands to activate calcium channels, the stimulatory effect of hGHRH and L-585 was significantly decreased in calcium depleted saline (Fig. 5A,B). In parallel run control experiments in normal Ca^{2+} HEPES, 88% of cells (70 of 80) responded to 10 μM hGHRH and L-585. Only 11% (9 of 81) of cells responded to 10 μM hGHRH in low Ca^{2+} HEPES. The average increase of $[\text{Ca}^{2+}]_i$ was 22 ± 7 nM, which is significantly less ($P < 0.05$) than that evoked with hGHRH in normal Ca^{2+} HEPES (52 ± 4 nM). In contrast, 52% (42 from 81) of the cells responded to 10 μM L-585 in low Ca^{2+} HEPES (Fig. 5B). The amplitude of L-585 evoked transient increase in $[\text{Ca}^{2+}]_i$ in low calcium HEPES was significantly smaller than in normal Ca^{2+} HEPES (67 ± 5 vs 34 ± 2 nM; $P < 0.01$), and the second, sustained phase of calcium increase was almost abolished (Fig. 5B). These results suggest that extracellular calcium has an important role in the effect of hGHRH and L-585 on GH secretory cells.

It has been demonstrated that GHRP-induced depolarization of somatotropes can activate voltage dependent calcium channels and result in an increase of $[\text{Ca}^{2+}]_i$ (Herrington & Hille 1994). To investigate the contribution of voltage dependent Ca^{2+} channels in calcium influx evoked by hGHRH and L-585, experiments were performed in the presence of nifedipine, an antagonist of the L-type calcium channel. In control cultures 10 μM hGHRH and 10 μM L-585 evoked a response in 94% of the cells (136 of 144). Application of 10 μM

hGHRH, in the presence of nifedipine, evoked a response in 88% of the cells (134 of 152) and Ca^{2+} transient in those cells was significantly decreased (55 ± 2 vs 25 ± 1 nM; $P < 0.01$; Fig. 5C). In the presence of 10 μM nifedipine, 91% of the cells (139 of 152) responded to 10 μM L-585. The average increase in $[\text{Ca}^{2+}]_i$ was significantly smaller (70 ± 3 vs 47 ± 2 nM; $P < 0.01$; Fig. 5C), and the duration of the sustained phase of calcium increase was brief.

To determine the role of Na^+ in hGHRH and L-585 induced calcium transients cultures were bathed in zero Na^+ solution (sodium was replaced with choline). Application of 10 μM hGHRH in normal HEPES solution in parallel run control experiments evoked an increase in $[\text{Ca}^{2+}]_i$ in 92% of the cells (126 of 137). In zero Na^+ HEPES, 71% of the cells (112 of 157) responded to 10 μM hGHRH, and the average amplitude of calcium increase was 19 ± 1 nM, a significantly smaller response compared to parallel run controls of 58 ± 2 nM ($P < 0.01$; Fig. 5D). Similarly, 84% of the cells (132 of 157) responded to 10 μM L-585 in zero Na^+ HEPES with 49 ± 3 nM increase in $[\text{Ca}^{2+}]_i$; the effect was significantly smaller compared with parallel run controls (61 ± 3 nM; $P < 0.01$; Fig. 5D). In zero Na^+ HEPES the effect of 50 mM K^+ on calcium transient was 40% of that recorded in normal HEPES (51 ± 2 vs 21 ± 1 nM; $P < 0.01$; Fig. 5D). These results further suggest an involvement of Na^+ dependent depolarization in calcium transients induced by both hGHRH and L-585.

Signal transduction pathways, phospholipase C (PLC), and adenylyl cyclase - cyclic adenosine 3,5-monophosphate (AC-cAMP), are activated by GH secretagogues

Results with the application of L-585 in low Ca^{2+} solution suggest that in L-585 induced GH secretion, extracellular Ca^{2+} has an important role but that intracellular Ca^{2+} has some role, too. The involvement of intracellular Ca^{2+} stores in hGHRH and L-585 action was further investigated in experiments with U73122, a selective inhibitor of phospholipase C (PLC) (Smith *et al.* 1990). Application of 10 μM U73122 for 10 min significantly decreased the effect of hGHRH on calcium transient (53 ± 3 nM, $n = 75$ vs 14 ± 1 nM, $n = 62$; $P < 0.01$). Inhibition of PLC by 10 μM U73122, also significantly reduced the effect of L-585 (77 ± 5 nM, $n = 75$ vs 12 ± 1 nM, $n = 110$; $P < 0.01$), implying an involvement of the PLC- IP_3 pathway (Fig. 6).

It has been shown that GHRH activates the cyclic adenosine 3,5-monophosphate pathway (Cheng *et al.* 1989). To determine whether L-585 activates this pathway, cell cultures were pretreated with SQ-22536, an adenylyl cyclase inhibitor (Tamaoki *et al.* 1993). SQ-22536 blocked the stimulatory effect of hGHRH and L-585 in a dose dependent manner. After 10 min application of SQ-22536 in concentrations of 50, 100, and 200 μM , the amplitude of $[\text{Ca}^{2+}]_i$ increase evoked by 10 μM hGHRH was decreased 51% ($P < 0.01$), 68% ($P < 0.01$), and 72% ($P < 0.01$), respectively (Fig. 7A). Pretreatment with SQ-22536 at a concentration of 50 μM decreased the stimulatory effect of L-585 to 41% ($P < 0.01$), while SQ-22536 in concentrations of 100 and 200 μM had more prominent effect and decreased stimulatory effect of L-585 to 55% ($P < 0.01$) (Fig. 7B).

Discussion

Porcine pituitary cells have a regulated secretory pathway utilized by secretory vesicles that fuse with the plasma membrane in response to a physiological stimulus such as GHRH or L-585 (Cho *et al.* 2002). Results from our experiments with simultaneous measurements of RHPA and calcium transients have shown that L-585 evoked an increase in $[\text{Ca}^{2+}]_i$ that coincided with GH release. The amplitude of $[\text{Ca}^{2+}]_i$ increase was greater after exposure to L-585 compared with hGHRH at the same micromolar concentration. GH secretion appears to be directly related to intracellular free calcium concentration (Lussier *et al.* 1991a) and our calcium imaging experiments indicate that L-585 was more potent than hGHRH in the rapid release of GH. Our earlier *in vivo* experiments in the pig have shown that L-585 was more efficacious than GHRH in releasing significantly greater amounts of circulating GH (Hickey *et al.* 1996). These *in vivo* experiments also showed that GHRH and L-585 have a synergistic effect on GH secretion (Hickey *et al.* 1996). Simultaneous application of hGHRH and L-585 in the present study, however, did not have an additive effect on intracellular calcium concentration in cultured porcine somatotropes.

Somatostatin abolished the effect of hGHRH and L-585, but at a lower concentration, it was more effective in suppressing the stimulatory effect of hGHRH compared with L-585. The effect of SS can be mediated by an inhibition of cAMP formation (Chen *et al.* 1996) or via decreased Ca^{2+} influx as a result of an increase in K^+ conductance and hyperpolarization

of somatotropes (Lussier *et al.* 1991b). GHS depolarize the plasma membrane of somatotropes by inhibiting K^+ channels, and have been suggested to behave as a functional antagonist of somatostatin (Smith *et al.* 1997).

GHRH receptor antagonist and a substance-P-analog with GHRH receptor antagonist properties did not influence the stimulatory effect of L-585, while they decreased and abolished the stimulatory effect of hGHRH on calcium transient, suggesting that L-585 and hGHRH act via different receptors on porcine somatotropes. Similarly, Smith *et al.* (1993) have shown that activity of L-692,429 can be blocked by both peptide and nonpeptidyl antagonists of GHRP-6, but not by an antagonist of GHRH. Contrary to our results on cultured porcine somatotropes, the SP analog in rats significantly reduced both GHRP-6- and L-692,429-stimulated GH response but had no effect in inhibiting GHRH-stimulated GH response (Cheng *et al.* 1994).

The depletion of extracellular calcium greatly diminished but did not abolish the stimulatory effect of hGHRH and L-585 on porcine somatotropes. This suggests that both GHRH and L-585 mobilize Ca^{2+} from intracellular stores, but Ca^{2+} influx has a major contribution to calcium transient. In somatotropes the major Ca^{2+} channels are the voltage-gated T- and L-types (Chen *et al.* 1990). Perfusion of porcine cells with nifedipine significantly decreased the effect of hGHRH and L-585. This indicates an involvement of L-type Ca^{2+} channels in calcium influx induced by hGHRH and L-585, and that influx of calcium is a crucial step in the action of GHS.

The stimulatory effect of hGHRH and L-585 was greatly attenuated in a zero Na^+ environment, suggesting that both ligands can depolarize somatotropes at least, in part, through sodium channels. GHRH transiently increases Na^+ and Ca^{2+} current while it decreases membrane K^+ conductance, which leads to depolarization and an influx of extracellular Ca^{2+} (Chen *et al.* 1994). Potassium channels may also be involved in the action of L-585, because electrophysiology studies showed that peptidomimetics blocked K^+ currents in somatotropes, resulting in a depolarization and electrical spiking to enhance Ca^{2+} entry through voltage gated channels (Smith *et al.* 1997). In our experiments the increase in $[Ca^{2+}]_i$ after applying K^+ was significantly decreased by nifedipine and in sodium free

solution, indicating that calcium and sodium channels were involved in depolarization of porcine somatotropes.

In this study, the few cells that responded to hGHRH in low calcium HEPES likely were low density (LD) cells. The first phase of L-585-evoked calcium increase resulted from intracellular Ca^{2+} mobilization, whereas the second phase represents calcium influx, because in low calcium HEPES, in nifedipine and in zero sodium HEPES, this second, sustained phase was almost abolished. In rats and pigs, it has been demonstrated that two morphologically and functionally distinct somatotrope subpopulations exist with LD and high density (HD) cells (Lindstrom & Savendahl 1996; Ramirez *et al.* 1999). In pigs blockade of Ca^{2+} influx with CoCl_2 reduced the GHRH-stimulated GH secretion in both LD and HD somatotropes, while depletion of thapsigargin-sensitive intracellular calcium stores only decreased the secretory response to GHRH in LD cells (Ramirez *et al.* 1999).

Pretreatment of porcine somatotropes with SQ-22536, an adenylyl cyclase inhibitor, decreased the stimulatory effect of hGHRH and L-585 on calcium transient in a dose-dependent manner, implying that the binding of hGHRH and L-585 to their receptors activates adenylyl cyclase. GHRP-2 applied to ovine somatotropes dose-dependently increased intracellular cAMP levels, whereas the GHRP-2-stimulated GH secretion was blocked by the inhibitor of adenylyl cyclase (MDL 12 330A) and by a cAMP binding antagonist (Rp-cAMP). GHRP-2 did not increase cAMP levels in rat somatotropes, suggesting the existence of several subtypes of GHS-R that are variably expressed in different species (Wu *et al.* 1996).

In our experiments U73122, a PLC inhibitor, decreased the effect of hGHRH on calcium transient, indicating the involvement of PLC/ IP_3 pathway in hGHRH action on porcine somatotropes. Ramirez *et al.* (1999) demonstrated that the adenylyl cyclase inhibitor (MDL-12,330A) abolished GHRH-stimulated GH release in both LD and HD subpopulations of porcine somatotropes, whereas U73122 only partially reduced this effect in LD cells. In the present investigation, U73122 decreased the transient increase in $[\text{Ca}^{2+}]_i$ evoked by L-585. Consistent with activation of this pathway were the observations that GHRP-6 and non-peptidergic GHSs increased PI turnover, and caused translocation of protein kinase C (PKC) (Adams *et al.* 1995, Mau *et al.* 1995). The activation and interplay of several pathways of

signal transduction mediate the effect of GHS in mobilizing Ca^{2+} , cAMP, protein kinase A and C, and phospholipase C (Smith *et al.* 1997, Muller *et al.* 1999).

In summary, L-585 in porcine somatotropes activates AC and PLC pathways, suggesting that it may act through different receptors that are coupled to G_s and G_q . Activation of these different signal transduction pathways mobilize calcium from internal stores during the first phase of the L-585-induced increase in $[\text{Ca}^{2+}]_i$. A second prolonged phase due to calcium influx results from somatotrope depolarization by L-585 acting on Na^+ and K^+ channels, and the activation of calcium channels through different second messengers. RHPA of cultured porcine somatotropes confirmed a connection between the L-585-evoked mobilization of calcium and GH release.

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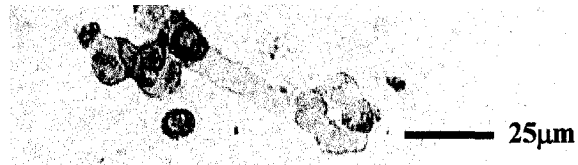
Figure 1 Morphological and functional identification of cultured porcine somatotropes:

A) Cells stained with anti-GH antibody in cultured porcine pituitary cells. Magnification 40 x.

B) The sequence of color images and kinetics of the $[Ca^{2+}]_i$ changes illustrate response of isolated pituitary cells to application of 10 μ M hGHRH, 10 μ M L-585 and 50 mM K^+ . The GH secretory cells (red x and red line in time-course histogram) responded to application of hGHRH, L-585 and 50 mM K^+ . The non GH-secretory cells responded to nonspecific stimulation of the 50 mM K^+ (green x and green line in time-course histogram) and nonexcitable cells (possible stellate cells) failed to respond to any of stimuli (blue x and blue line in time-course histogram).

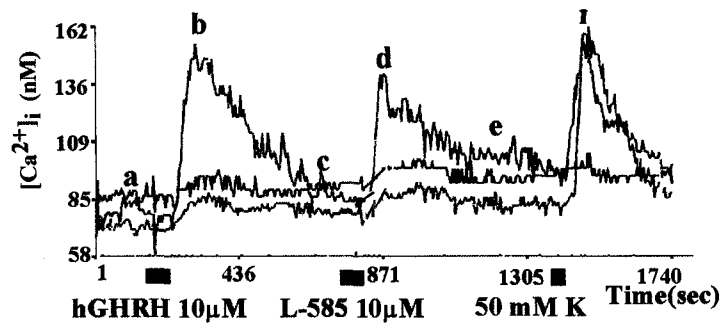
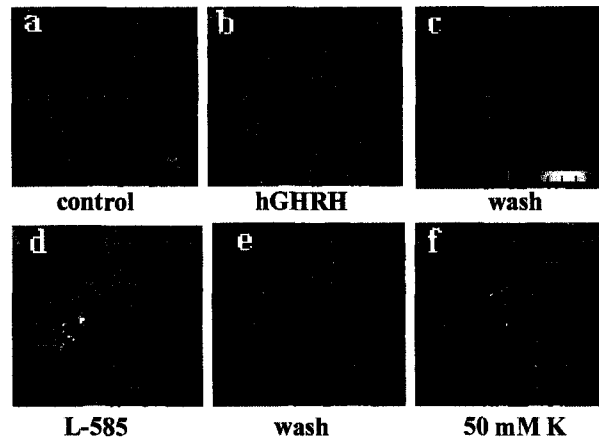
C) Calcium transient induced by L-585 coincides with release of GH. Bath application of L-585 induced calcium transient in one of two cells illustrated in a and b images and in time course histogram in d. Phase contrast images in c illustrate formation of plaque only around the cell that responded with calcium transient to L-585 application in b.

A



GH positive cells

B



C

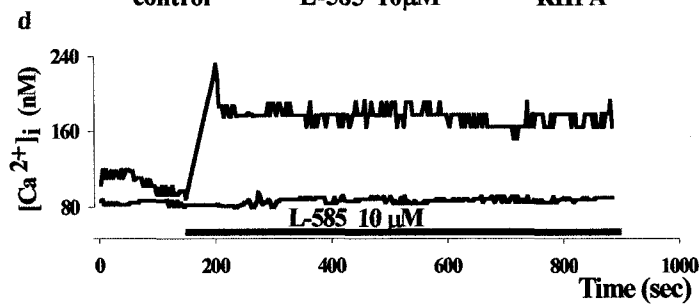
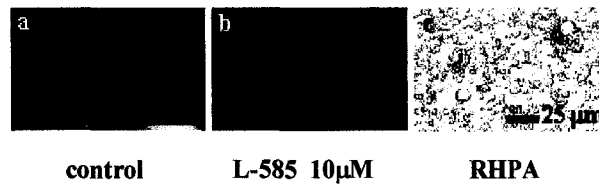


Figure 2 Treatment with L-585 increases GH secretion from isolated porcine somatotropes determined by reverse hemolytic plaque assay (RHPA):

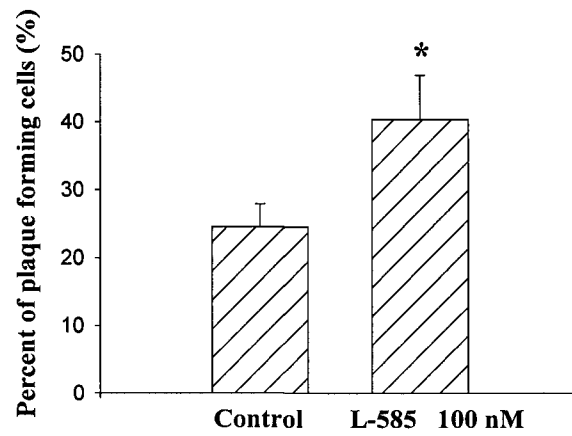
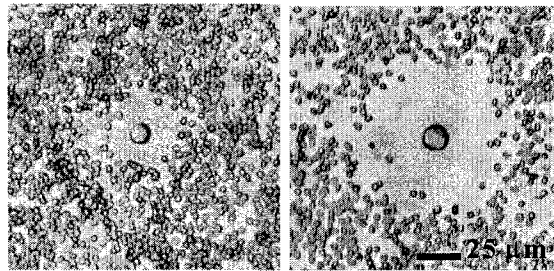
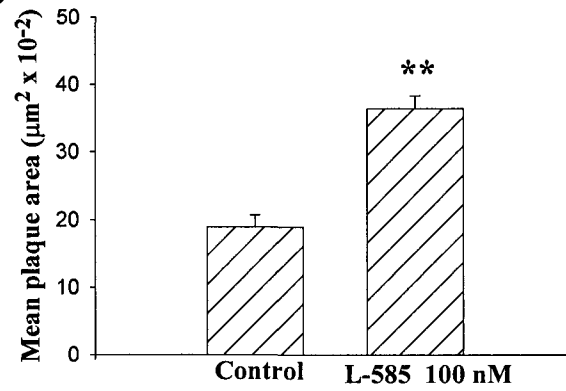
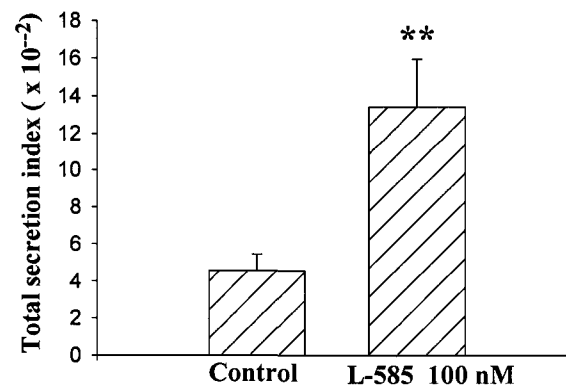
A) Percentages of GH-plaque forming cells (% PFC) in dispersed anterior pituitary cells when exposed for 3 h to vehicle (control) or 100 nM L-585. The results are expressed as the mean \pm SEM for three separate plaque assays. Two hundred pituitary cells were counted per assay (*, $P < 0.05$ vs vehicle).

B) The mean plaque areas (MPA) of somatotropes in RHPA described in A are shown on phase contrast images (a) and on histograms (b).

a) Monolayers prepared from the same protein-A labeled ovine erythrocytes (small cells) and somatotropes (large cells) derived from 2-day old pigs. The cultures were treated with control medium and L-585 100 nM. The cultures were incubated with GH-antibody (1:60) for 3 h, exposed to complement (1:40) for 50 min, then fixed in 1% glutaraldehyde for 50 min. Phase contrast images show the increase in size of plaques (pale areas in the monolayer of red blood cells) formed by somatotropes (oval cells in the center of each plaque) treated with L-585.

b) The MPA of somatotropes are expressed as the mean \pm SEM of the mean plaque areas of 100 plaque forming cells from 3 separate plaque assays (**, $P < 0.01$ vs vehicle).

C) The total secretion index (TSI) of somatotropes in RHPA described in A, is calculated by multiplying PFC and MPA (**, $P < 0.01$ vs vehicle).

A**B****a****b****C**

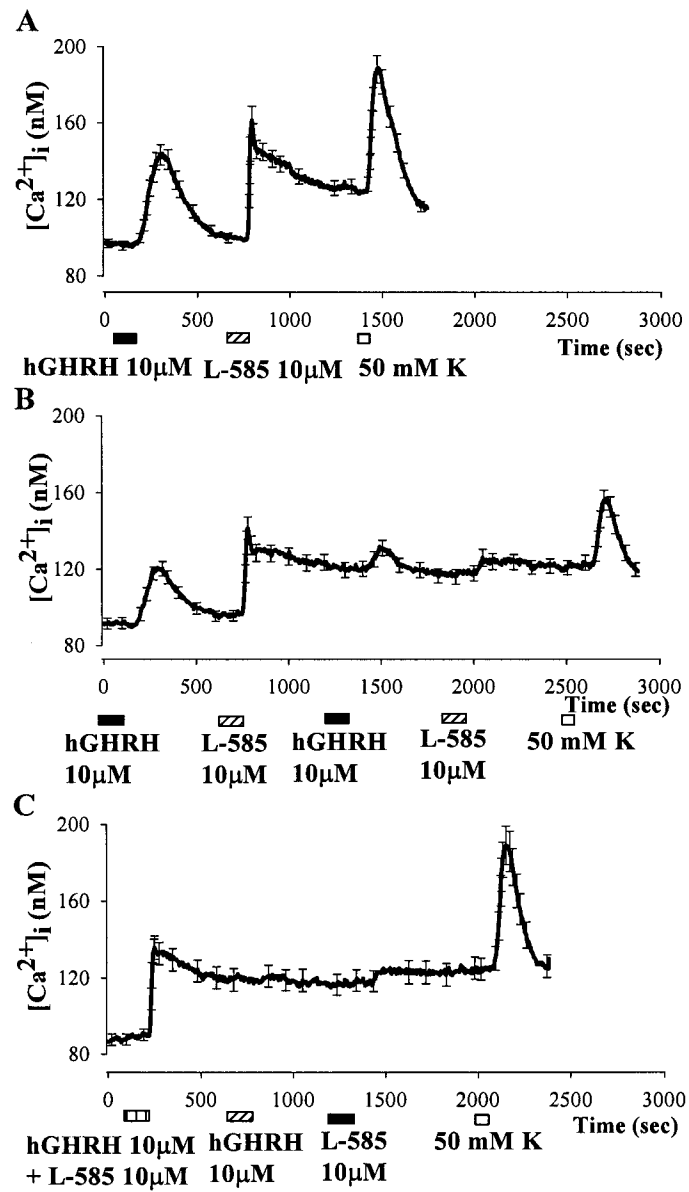


Figure 3 hGHRH and L-585 induce calcium transient in isolated porcine somatotropes:

A) Increase in $[Ca^{2+}]_i$ after application of 10 μ M hGHRH, 10 μ M L-585, and 50 mM K^+ ($n = 51$).

B) The second application of 10 μ M hGHRH and 10 μ M L-585 did not have an additive effect on the $[Ca^{2+}]_i$ increase ($n = 57$).

C) Simultaneous application of 10 μ M hGHRH and 10 μ M L-585, did not cause an additive effect on the increase in $[Ca^{2+}]_i$ ($n = 58$).

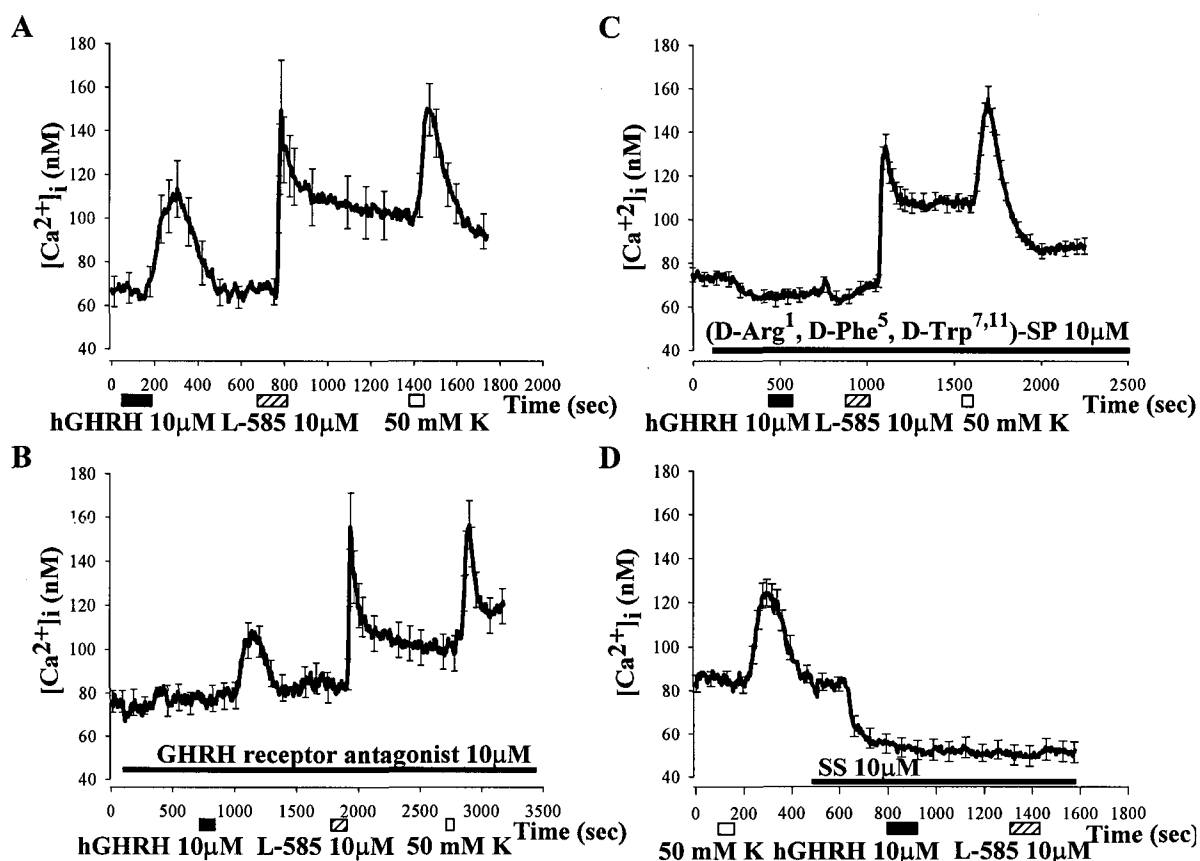


Figure 4 hGHRH and L-585 increased $[Ca^{2+}]_i$ in isolated porcine somatotropes acting through different receptors:

A) Control effect of 10 μ M hGHRH, 10 μ M L-585, and 50 mM K⁺ on calcium transient in isolated porcine somatotropes ($n = 21$).

B) Application of GHRH receptor-antagonist, Phenylac-Tyr¹, D-Arg², p-chloro-Phe⁶, Homoarg⁹, Tyr (Me)¹⁰, Abu¹⁵, Nle²⁷, D-Arg²⁸, Homoarg²⁹-GRF (1-29) amide (human), decreased stimulatory effect of 10 μ M hGHRH, while it had no influence on L-585 effect ($n = 30$).

C) Substance P analog abolished only the effect of hGHRH, but not that of L-585 ($n = 44$).

D) Application of 10 μ M SS decreased $[Ca^{2+}]_i$ and blocked the stimulatory effect of hGHRH and L-585 ($n = 10$).

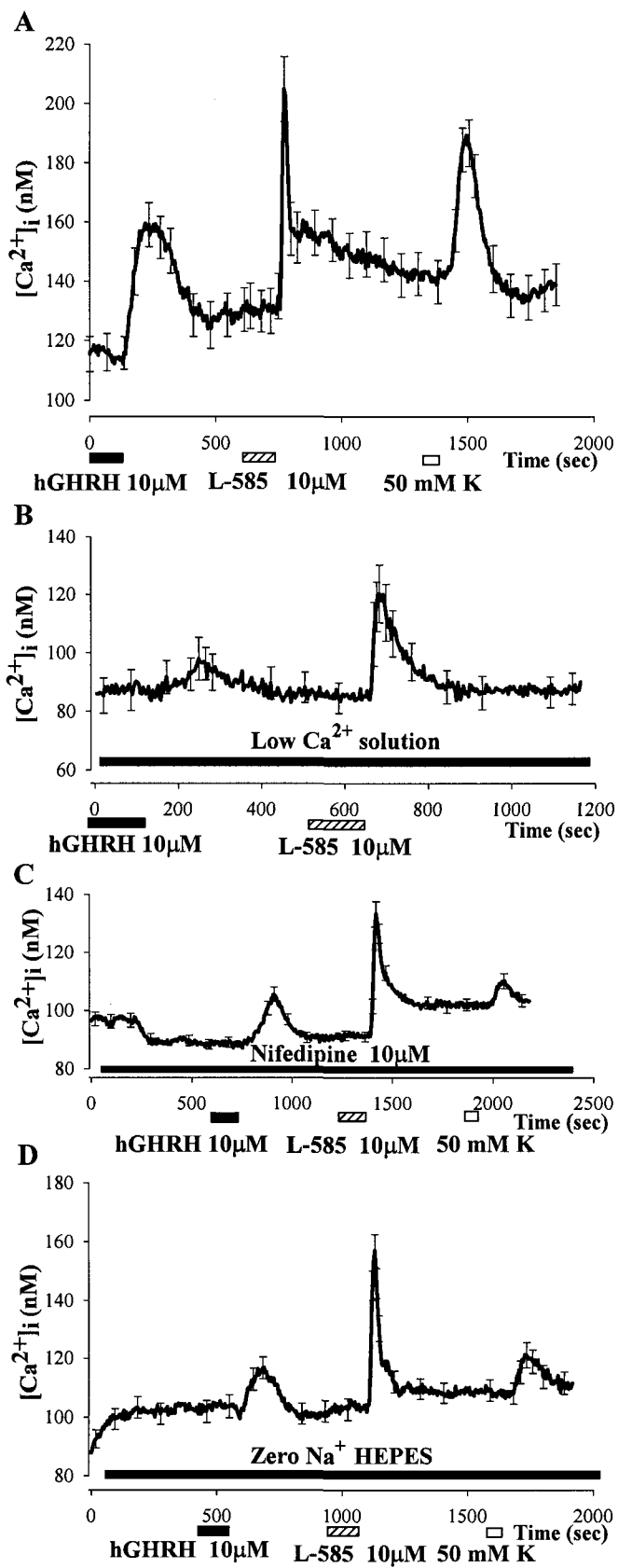
Figure 5 Influx of calcium is involved in hGHRH and L-585 induced calcium transient in isolated porcine somatotropes:

A) Control effect of 10 μM hGHRH, 10 μM L-585, and 50 mM K^+ on calcium transient in isolated porcine somatotropes ($n = 40$).

B) Depletion of Ca^{2+} from bathing solution dramatically decreased the stimulatory effect of hGHRH and L-585 on isolated somatotropes ($n = 5$).

C) Perfusion of culture with 10 μM nifedipine, a blocker of L-type Ca channels, significantly decreased stimulatory effect of hGHRH, L-585, and 50 mM K^+ on isolated somatotropes ($n = 70$).

D) Removal of Na^+ ions from the bathing solution significantly decreased the effect of 10 μM hGHRH, 10 μM L-585, and 50 mM K^+ on calcium transient ($n = 37$).



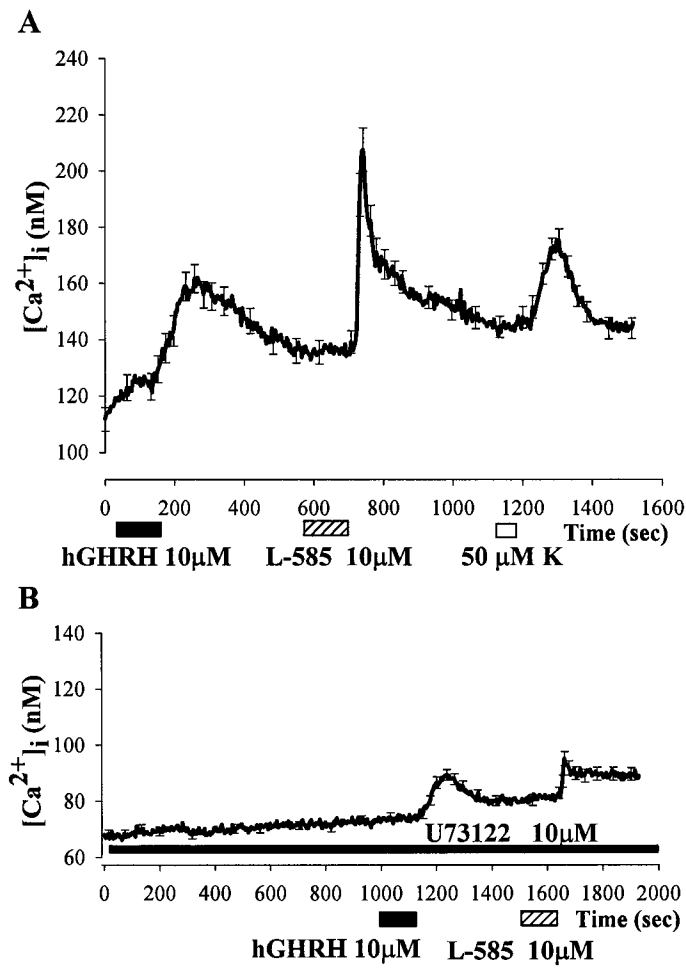


Figure 6 PLC-IP₃ pathway is involved in stimulatory effect of hGHRH and L-585 on cultured porcine somatotropes:

A) Control effect of 10 μ M hGHRH, 10 μ M L-585, and 50 mM K⁺ on calcium transient in isolated porcine somatotropes ($n = 36$).

B) Pretreatment of the cultured porcine pituitary cells with 10 μ M U73122 significantly decreased the stimulatory effect of hGHRH and L-585 ($P < 0.01$; $n = 18$).

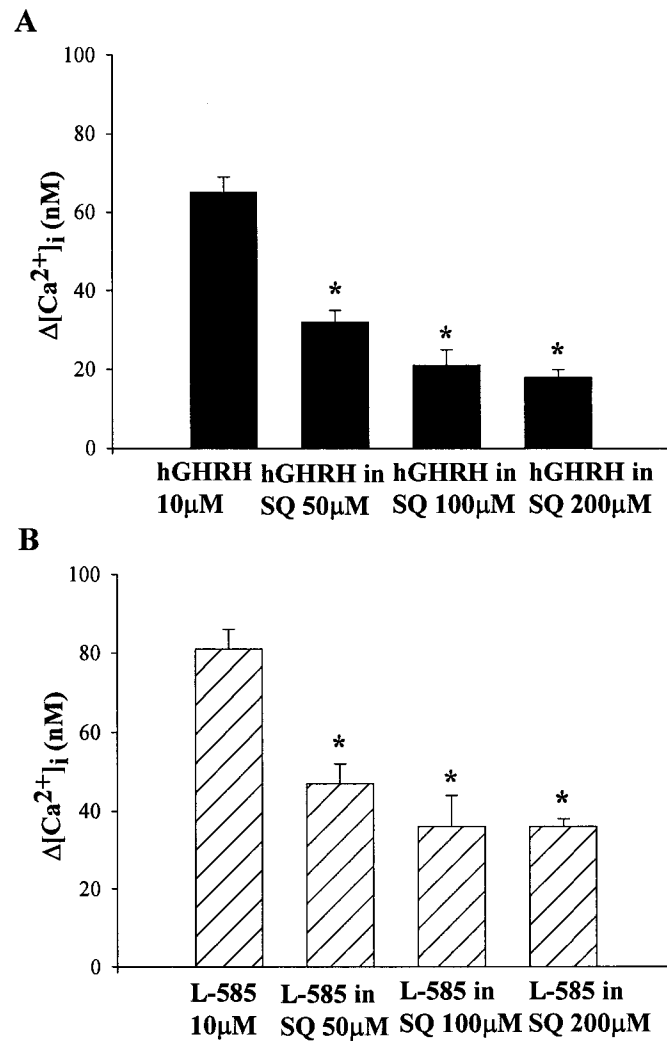


Figure 7 Application of SQ-22536 (SQ), an adenylyl cyclase inhibitor, dose dependently blocked the stimulatory effect of hGHRH and L-585:

A) Dose dependent effect of SQ on hGHRH induced calcium transient in isolated porcine somatotropes [* , $P < 0.01$ control (hGHRH) vs hGHRH+SQ].

B) Dose dependent effect of SQ on L-585 induced calcium transient in isolated porcine somatotropes [* , $P < 0.01$ control (L-585) vs L-585+SQ].

CHAPTER 3. STRUCTURE AND DYNAMICS OF THE FUSION PORES IN LIVE GH-SECRETING CELLS REVEALED USING ATOMIC FORCE MICROSCOPY

A paper published in *Endocrinology* (2002)

Sang-Joon Cho, Ksenija Jeftinija, Aleksandra Glavaski, Srdija Jeftinija, Bhanu P. Jena and
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Abstract

Earlier studies in live pancreatic acinar cells identified new cellular structures at the cell plasma membrane called 'pits' and 'depressions', where membrane-bound secretory vesicles dock and fuse to release vesicular contents. In the current study, using atomic force microscopy we identify similar structures at the plasma membrane of GH-secreting cells of the pituitary and implicate their involvement in hormone release. Pits containing 100-200 nm in diameter depressions or fusion pores were identified in resting GH-secreting cells. Following stimulation of secretion the size of depression enlarged and gold-tagged GH antibody were found to bind to the pit structures in the stimulated GH cells. This study documents for the first time the presence of these structures and their involvement in secretions in a neuroendocrine cell.

Introduction

Until recently (1 and unpublished data), our understanding of secretory vesicle fusion at the cell plasma membrane was obtained from morphological (2-5), electrophysiological (6-11), and biochemical studies (2, 12-15) all suggesting the presence of 'fusion pores' at the cell plasma membrane. Using atomic force microscopy (AFM), the structure and dynamics of the 'fusion pore' were first revealed and examined in live pancreatic acinar cells (1 and unpublished data). In live resting pancreatic acinar cells, 'pits' measuring 0.5-2 μm and

containing 3-20 'depressions' of 100-180 nm diameter were identified only at the apical region of these cells where membrane-bound secretory vesicles are known to dock and fuse. Following stimulation of secretion, only 'depression' enlarged and returned to resting size following completion of secretion. Exposure of acinar cells to cytochalasin B, a fungal toxin that inhibits actin polymerization, resulted in a 50-60% loss of stimutable amylase secretion. A significant decrease in depression diameter was also observed in acinar cells exposed to the fungal toxin. To determine if similar structures are present in neuroendocrine cells, the GH-secreting cell of the pig pituitary was studied using AFM. Results from this study demonstrate the presence of pits and depressions in GH-secreting cells of the pituitary and their involvement in hormone release.

Material and Methods

Experimental animals

Yorkshire pigs, raised at the Iowa State University Animal Nutrition Farm, were used for these experiments. Newborn pigs, 1-8 days of age, were killed with electricity and decapitated. Pituitary glands were immediately removed and collected in cold sterile EBSS solution (4 °C). Posterior lobes were discarded and anterior lobes were transferred to a sterile cold (4 °C) MEM-0.1% BSA medium. Animal care and experimental protocols were in accordance with the guidelines and approval of the Iowa State University Committee on Animal Care.

Isolation and stimulation of GH-secreting cells

The protocol used for establishing primary cell cultures from pig anterior pituitary gland is a modification of procedure used for establishing neuronal cultures (16). In brief, tissue was incubated with 2.5% papain solution for 40min at 37 °C. After the incubation, tissue was mechanically dispersed in a presence of 1 ml of culture medium by triturating through a 1-ml fire-polished glass pipette 8-10 times and plated on the poly-L-lysine (1 mg/ml, MW 100,000; Sigma) coated dishes. Cells were incubated overnight at 37 °C in a humidified 5% CO₂/95% air atmosphere to allow them to adhere. Culture medium consisted of Eagle's MEM supplemented with 10% FBS and 40 mM glucose, 2 mM L-glutamine, 1 mM pyruvate, 14

mM sodium bicarbonate and penicillin/streptomycin. Cells in PBS, were stimulated with 20 μ M of the GH secretagogue L-692,585.

Assay of GH-secretion using Reverse Hemolytic Plaque Assay

Reverse Hemolytic Plaque Assays (RHPA) were performed using a minor modification of the method of (17). Staphylococcus protein A was coupled to ovine red blood cells using 0.1% CrCl_3 . Isolated pituitary cell were incubated for 48 h to allow cells to acclimate. Cells were then treated with trypsin (2.5 mg trypsin in 10 ml MEM-0.1% BSA) to detach the cells from the culture dish. After gentle trituration using a fire-polished glass pipette, cells were separated by centrifugation ($1500 \times g$ for 10 min), washed twice in MEM-0.1% BSA. A cell concentration of 2.5×10^5 cells/ml was used in RHPA experiments. A 1:1 pituitary cell to 50% protein A-labeled erythrocyte solution were mixed, and infused into Cunningham slide chambers. The chambers with the cell infusion were preincubated for 50 min at 37 °C in 5% CO_2 -95% air. Slides were pretreated with poly-L-lysine (0.1 mg/ml; MW 100,000), to ensure cell adhesion. After incubation, the chambers were rinsed with DMEM-0.1% BSA, followed by addition of anti porcine GH raised in monkey (1:60 dilution in DMEM-0.1% BSA), in the presence and absence of 100 nM L692,585. After 3 h incubation, plaque formation was initiated by infusing, guinea pig complement (1:40 in DMEM-0.1% BSA). The reaction was terminated after 50 min by infusion of 1% glutaraldehyde. The secretion index is represented by the size of the plaque. These isolated cells were used in our AFM studies.

Atomic Force Microscopy

'Pits' and 'depressions' at the plasma membrane in live and fixed GH secreting, cells ($n = 24$) in PBS pH 7.5 were imaged by the AFM (Bioscope III, Digital Instruments) using- both contact and tapping mode. All images presented in this manuscript were obtained in the "tapping" mode in fluid, using silicon nitride tips with a spring constant of 0.06 Nm^{-1} , and an imaging force of $<400 \text{ nN}$. Images were obtained at line frequencies of 1 Hz, with 512 lines per image, and constant image gains. Topographical dimensions of 'pits' and 'depressions' at the cell plasma membrane were analyzed using the software nanoscopeIIIa4.43r8 supplied by Digital Instruments.

Immunogold AFM

Following stimulation of secretion using 20 μ M L-092,585, live GH secreting cells of the pituitary were exposed to 1:200 dilution of Growth Hormone-specific antibody, and 30 nm gold conjugated secondary antibody, washed in PBS, prior to AFM imaging in PBS at room temperature.

Cell Fixation and immunogold localization

Following stimulation of secretion using 20 μ M L-692,585, live GH secreting cells were fixed for 30 min using ice-cold 2.5% paraformaldehyde in PBS. Cells were then washed in PBS, followed by labeling with 1:200 dilution of Growth Hormone-specific antibody, and 30 nm gold conjugated secondary antibody, washed in PBS, prior to AFM imaging in PBS at room temperature.

Results and Discussion

Reverse hemolytic plaque assay on isolated GH secreting cells of the pig pituitary demonstrated GH release following exposure to L-692,585 (Fig. 1). Examination of resting and stimulated GH cells demonstrates no detectable changes following fixation (Fig. 2). Examination of resting GH cells revealed the presence of 'pits' and 'depressions' at the plasma membrane. Depressions in resting cells measure 154 ± 4.5 nm (mean \pm SE). However, following exposure of GH cells to the secretagogue L-692,585, a 40% increase in the size (215 ± 4.6 nm; $P < 0.01$) of 'depressions' is demonstrated (Fig. 3, 4). When stimulated live cells were exposed to 30 nm-gold-tagged GH-antibody, gold particles were found to decorate 'pit' and 'depression' structures (Fig. 3). Aldehydes fixation of biological samples is known to result in decreased elasticity and increased hardness (18, 19 and *personal observation*). Since force spectroscopy is used to image objects using the AFM, less elastic samples are better resolved. From studies using that pancreatic acinar cells (unpublished), it was determined 1-h treatment of ice-cold 2.5% paraformaldehyde in phosphate buffered saline, pH 7.5, was ideal in retaining the structural integrity of 'pits' and 'depressions'. No detectable changes were identified in live cells following fixation. To obtain high resolution images of the cells, and the 'pits' and 'depressions' and to determine

the distribution of 30 nm GH-immunogold labeling, stimulated GH cells were fixed following immunogold labeling. In conformation with our observation in live GH cells, AFM images of the immunolabeled fixed cells, demonstrate specific localization of immunogold at 'depressions' (Fig. 4), implicating them to be secretory sites at the cell plasma membrane. In agreement with earlier studies in pancreatic acinar cells, the GH-secreting neuroendocrine cells of the pituitary demonstrate the presence of 'pits' and 'depressions' at the plasma membrane, where secretory vesicles dock and fuse to release vesicular contents. The presence of 'pits' and 'depressions' in both exocrine and neuroendocrine cells suggests that these structures may be universal to secretory cells, where exocytosis occurs (Fig. 5).

Acknowledgments

All experiments in this report were performed following standards established by the American Welfare Act and NIH Guide for the Care and Use of Laboratory Animals, Publication 85-23. This is J- 19620 of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa (Projects 3901 and 2273, the last contributing project to North Central Regional Project NC-113), L-692,585 was a gift from G. J. Hickey, Merck Research Laboratories, Rahway, NJ. This work was supported by NIH Grants DK56212, and NS39918 (to B.P.J.) and NIH Fellowship DK60368 (to S.J.C.).

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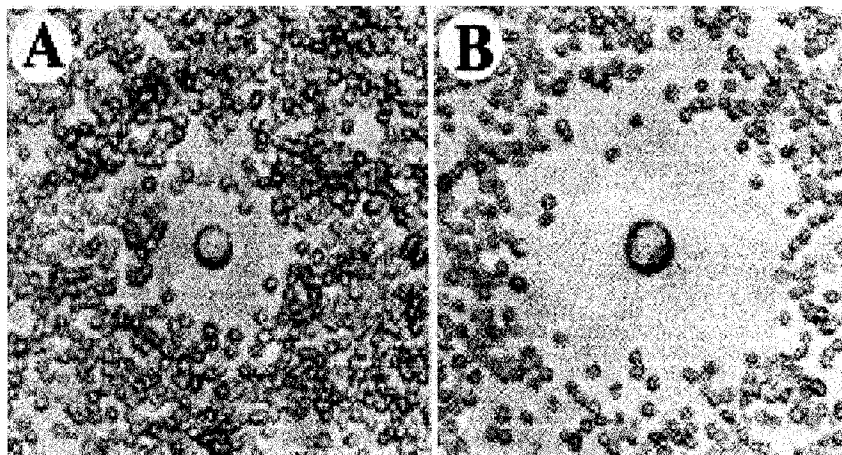


Fig. 1. Light microscopy revealing the extent of growth hormone release from isolated GH cells of the pituitary gland, in a typical Reverse Hemolytic Plaque Assay. Larger the plaque (clear area), more the release. Note the presence of a small plaque in a resting GH cell (A), compared to a L-692,585 stimulated cell (B).

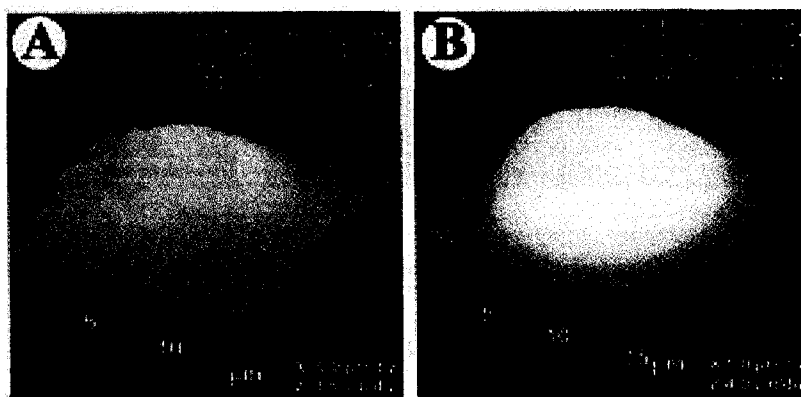


Fig. 2. Atomic Force Microscopy (AFM) performed on whole GH cells. AFM. micrograph of a live (A) and fixed (B) GH secreting cells, imaged in fluid (PBS).

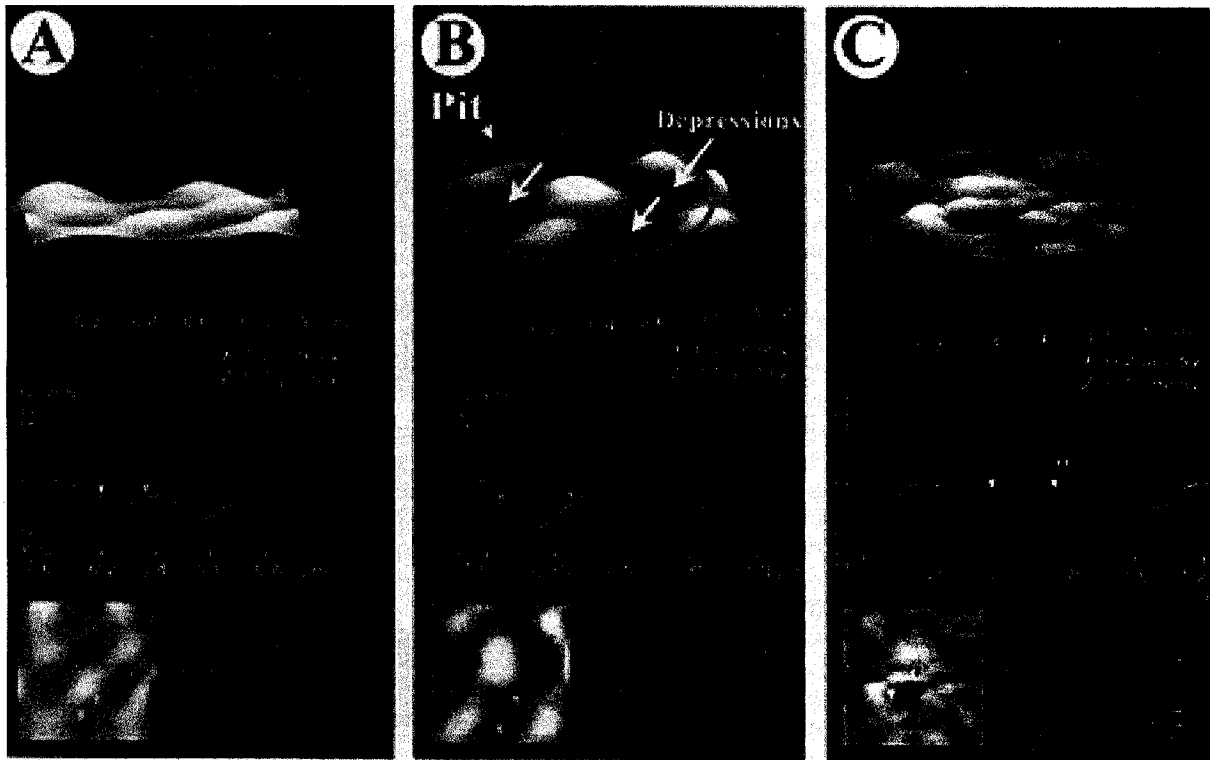


Fig. 3. High resolution Atomic Force Microscopy (AFM) performed on live GH secreting cells, showing a single 'pit' with 3-4 'depressions' (*top panel*). The scan line in the *middle panel* depicts the relative height and depth between arrows, and the *bottom panel* is section analysis of two 'depressions' showing their relative depth and diameter. (A) in the resting cell and (B) when the same cell is GH-secretagogue stimulated, and (C) following exposure to 30 nm-gold-tagged GH-antibody following the stimulation. Note the enlargement of the 'depressions', following stimulation of secretion [(A) compared with (B)] and decoration of the 30 nm-gold-tagged GH-antibody (C) at the edges and one within a 'depressions'.

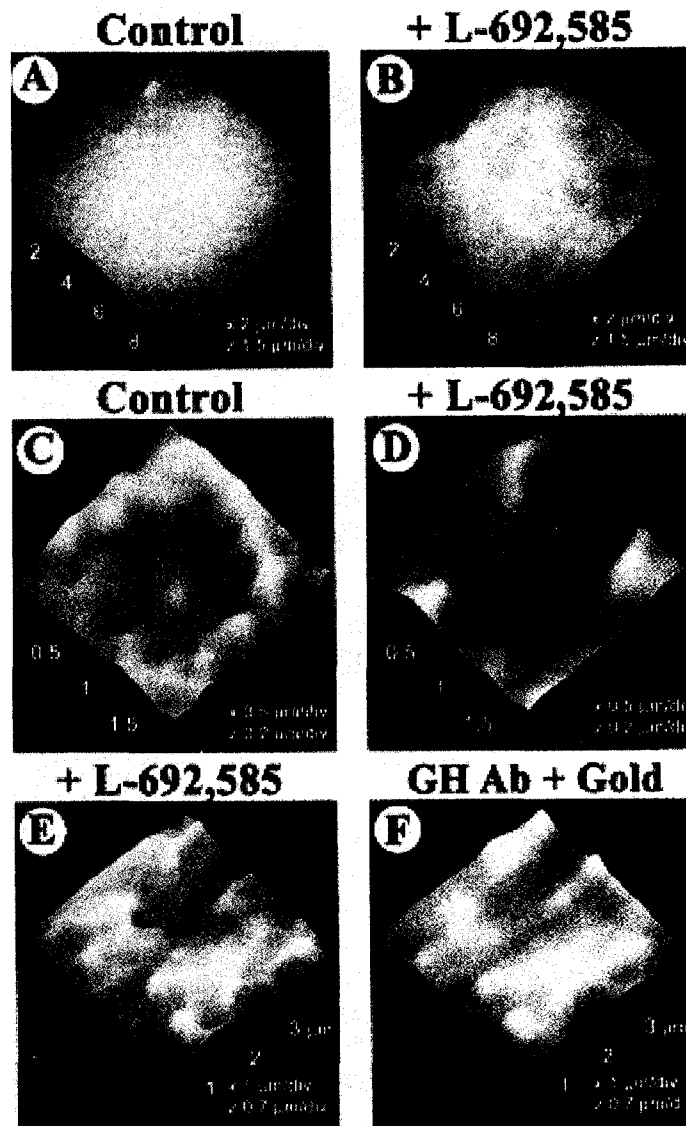


Fig. 4. High resolution Atomic Force Microscopy (AFM) performed on resting and stimulated GH secreting cells, after fixation. Note a large area of a cell surface in resting (A) and in a stimulated (B) cell. AFM micrographs of a 'pit' with 'depressions' in resting cell (C), again clearly demonstrating the enlargement of 'depressions' or fusion pores following stimulation of secretion (D). Exposure of 'pits' in a stimulated cell (E) to 30 nm gold-tagged GH-antibody, results in binding of released growth hormone at the site to 30 nm gold-tagged GH-antibody (F). Note the loss of fusion pores due to large amounts of gold-tagged antibody binding at these sites.

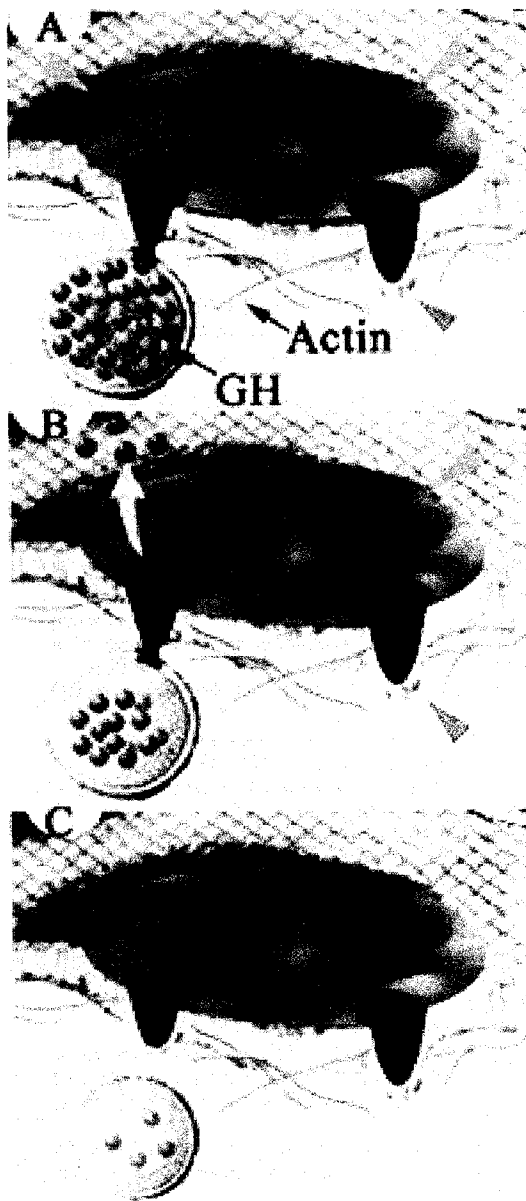


Fig. 5. Schematic diagrams depict cross sectional views at cell plasma lipid membrane of a 'pit' (green arrowhead) and 'depressions' (red arrow head) as well as a vesicle which contains hormone (GH), within the cell cytoplasm. Immediately following GH-secretagogue stimulation, the secretory vesicle containing hormone docks and fuses with the 'depression' (A) and then releases hormone (B) through the fusion pores of the depression' (yellow arrow). After hormone release from the vesicle (C), the 'depression' becomes smaller and the vesicle returns to the cellular cytoplasm.

CHAPTER 4. STIMULATORY EFFECT OF GHRELIN ON ISOLATED PORCINE SOMATOTROPES

Paper submitted to *Neuroendocrinology*

Aleksandra Glavaski-Joksimovic, Ksenija Jeftinija, Colin G. Scanes, Lloyd L. Anderson, and
Srdija Jeftinija

Absrtact

Research on the mechanism for growth hormone secretagogue (GHS) induction of growth hormone (GH) secretion led to the discovery of GH secretagogue receptor (GHS-R) and later to ghrelin, an endogenous ligand for GHS-R. The ability of ghrelin to induce an increase in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in somatotropes was examined in dispersed porcine pituitary cells using a calcium imaging system. Somatotropes were functionally identified by application of human growth hormone releasing hormone (hGHRH). Ghrelin increased $[\text{Ca}^{2+}]_i$ in a dose dependent manner in 98 % of the cells that responded to hGHRH. In the presence of (D-Lys³)-GHRP-6, a specific receptor antagonist of GHS-R, the increase in $[\text{Ca}^{2+}]_i$ evoked by ghrelin was decreased. Pretreatment of cultures with somatostatin, or neuropeptide Y, reduced the ghrelin-induced increase of $[\text{Ca}^{2+}]_i$. The stimulatory effect of ghrelin on somatotropes was greatly attenuated in low calcium saline and blocked by nifedipine, a L-calcium channel blocker, suggesting involvement of calcium channels. In a zero Na^+ solution, the stimulatory effect of ghrelin on somatotropes was decreased, suggesting that beside calcium channels, sodium channels are also involved in ghrelin-induced calcium transients. Either SQ-22536, an adenylyl cyclase (AC) inhibitor, or U73122, a phospholipase C (PLC) inhibitor, decreased the stimulatory effects of ghrelin on $[\text{Ca}^{2+}]_i$ transient indicating the involvement of AC-cyclic adenosine monophosphate (cAMP) and PLC- inositol 1,4,5-trisphosphate IP_3 pathways.

The nonpeptidyl GHS, L-692,585 (L-585), induced changes in $[\text{Ca}^{2+}]_i$ similar to those observed with ghrelin. Application of L-585 after ghrelin, did not have additive effect on

[Ca²⁺]_i. Pre-application of L-585 blocked the stimulatory effect of ghrelin on somatotropes. Simultaneous application of ghrelin and L-585 did not cause an additive increase in [Ca²⁺]_i. Our results suggest that the action of ghrelin and synthetic GHS closely parallel each other, in a manner that is consistent with an increase of hormone secretion.

Introduction

GROWTH HORMONE (GH) secretion from the pituitary gland is controlled by two hypothalamic peptides – stimulatory - GH releasing hormone (GHRH) and inhibitory - somatostatin (SRIH). GH participates in its own rhythmic secretion through feedback action on GHRH and SRIH neurons [1]. Peptidyl and nonpeptidyl compounds, known as growth hormone secretagogues (GHS), can also stimulate GH secretion both *in vivo* and *in vitro* [2]. GH releasing peptides (GHRP) were the first generation of GHS [3, 4]. Later nonpeptidyl classes of benzolactam and spiroindolamine GHS with better oral bioavailability and pharmacokinetics properties were developed [5]. The mechanism of action of GHS is not fully established. There is both a direct effect on the anterior pituitary gland and an indirect effect on the hypothalamus. The latter suggests that exogenous GHS may induce the release of another hypothalamic factor with GH-releasing capabilities (U-factor) [2, 3]. The direct effect of GHS on GH secretion is through an interaction with a specific receptor named GHS-R. These are distinct from the GHRH receptor [6]. The GHS-R, a typical G-protein-coupled seven-transmembrane receptor, has been cloned in pigs, humans, and rats [7, 8].

Ghrelin, an endogenous ligand for GHS-R, is a 28-amino acid peptide with an n-octanoyl modification at Ser³ residue that is essential for its biological activity [9, 10]. Ghrelin is predominantly produced by the stomach, whereas lower amounts are derived from hypothalamus and various peripheral tissues [9, 11]. Ghrelin receptors (GHS-R) are widely distributed e.g. in the pituitary, hypothalamus, stomach, heart, blood vessels, lung, pancreas, intestine, kidney, adipose tissue and immune system [11, 12]. This suggests that ghrelin can have peripheral and central effects in addition to having a powerful effect on the secretion of GH. Moreover, ghrelin signals directly to the hypothalamic regulatory nuclei those control energy homeostasis, and, therefore, might regulate growth process in an integrated manner [13].

Ghrelin has been demonstrated to induce GH secretion from the pituitary *in vivo* and *in vitro* [9, 14]. Recently ghrelin, has been shown to stimulate GH release in humans in a dose dependent manner [15, 16, 17]. The effects of ghrelin exhibit a similar time course for GH release to that with other GHS [15]. Several studies confirm that the actions of ghrelin and peptidyl GHS closely parallel each other [16, 18].

The present study examined the signal transduction pathways of ghrelin in isolated porcine somatotropes and the interaction between ghrelin and nonpeptidyl GHS, L-692,585 (L-585). An understanding of the molecular mechanisms by which ghrelin and GHS modulate GH secretion is of particular interest in the regulation of GH for muscle accretion and somatic growth.

Materials and Methods

Chemicals

Fetal bovine serum (FBS), horse serum (HS), minimum essential medium (MEM), Dulbecco's modified Eagle's medium (DMEM) and Earle's balanced salt solutions (EBSS) were purchased from Gibco-Invitrogen Co. (Carlsbad, CA, USA). HEPES, ethyleneglycol-bis-(β -aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), papain solution, trypsin-inhibitor, penicillin-streptomycin solution, poly-L-lysine, L-cysteine, L-glutamine, sodium pyruvate, bovine serum albumin (BSA) and nifedipine were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Paraformaldehyde was obtained from Fisher Chemical (Fair Lawn, NJ, USA). Fura 2/AM and Pluronic F-127 were purchased from Molecular Probes (Eugene, OR, USA). ABC kit was obtained from Vector Laboratories (Burlingame, CA, USA). Somatostatin-14 (SRIH), Porcine Neuropeptide Y (NPY) and (D-Lys³)-GHRP-6 were obtained from Bachem California, Inc. (Torrance, CA, USA). U73122 and SQ-22536 were purchased from Biomol Research Laboratories (Plymouth Meeting, PA, USA). Ghrelin (human) was purchased from American Peptide Company, Inc. (Sunnyvale, CA, USA). Human GHRH and anti-porcine GH antibody were gifts from Dr A F Parlow, NIDDK National Hormone and Pituitary Program (Torrance, CA, USA). L-692,585 (L-585) was a gift from Dr G J Hickey, Merck Research Laboratories, Rahway, NJ, USA.

Experimental animals

Yorkshire pigs, raised at the Iowa State University Animal Nutrition Farm, were used for these experiments. Animal care and experimental protocols were in accordance with the guidelines and approval of the Iowa State University Committee on Animal Care.

Preparation of cell cultures

Newborn pigs, 1-8 days of age ($n = 26$), were killed with electric shock and decapitated. Pituitary glands were immediately removed. The pituitary glands were collected in cold sterile EBSS solution (4°C). Anterior lobes were transferred to a sterile cold (4 °C) MEM-0.1 % BSA medium. Primary cell cultures from neonatal anterior pituitary gland were established using a modified method of Huettner & Baughman [19]. Tissues from two animals were incubated 50 min at 37 °C in 2 ml of EBSS-papain solution (1.54 mg/ml). After incubation, the tissue was rinsed with EBSS solution and incubated for 5 min in trypsin-inhibitor solution (1 mg/ml). After being rinsed, once with EBSS solution and once with DMEM-0.1 % BSA medium, the tissue was mechanically dispersed in DMEM-0.1 % BSA medium by triturating through a firepolished glass pipette. The undigested tissue was allowed to sediment. The supernatant containing cells was removed and filtered through sterile filter. Cells were plated onto poly-L-lysine (0.1 mg/ml; 100,000 KD) coated glass coverslips (at a density of 2×10^5 cells). Cells were allowed to attach, and then after 3-4 h DMEM-0.1 % BSA medium was exchanged with DMEM medium supplemented with 10 % horse serum and 1 ml penicillin-streptomycin solution per 100 ml medium. Cultures were maintained at 37 °C in a humidified 5 % CO₂/95 % air atmosphere. Studies examining effects of ghrelin on changes in intracellular calcium concentrations $[Ca^{2+}]_i$ in somatotropes were carried out after 2 days in culture. The presence of somatotropes was confirmed by immunocytochemical methods.

Immunocytochemistry staining

After fixation with 4 % paraformaldehyde for 30 min at room temperature, cells were incubated for 30 min in a 50 % goat serum solution containing 1 % BSA and 100 mM L-lysine to block nonspecific binding and 0.4 % Triton X-100 to permeabilized the membrane.

To stain the somatotropes, cultures of anterior pituitary gland were incubated in polyclonal anti-porcine GH antibody (dilution 1:50,000). Antibody visualization was accomplished by using a Vectastain ABC kit (Vector) and the nickel-enhanced 3-3' diaminobenzidine method [20].

Intracellular calcium imaging

The effect of secretagogues on intracellular calcium concentrations $[Ca^{+2}]_i$ was evaluated by ratiometric imaging techniques [21]. Cells were loaded with Fura 2-AM for 40-60 min at 37 °C. 1 µl of 25 % (w/w) of Pluronic F-127 was mixed with 4 nM of AM ester to aid solubilization of the ester into aqueous medium. Coverslips containing pituitary cells were washed with HEPES-buffered solution and further incubated for 10 min at 37 °C to allowed deesterification of Fura 2-AM. All image processing and analysis were performed using an Attoflour system (Atto Bioscience) with Zeiss microscope. Background subtraction and ratio images were used to calculate the $[Ca^{+2}]_i$ according to Equation 5 of Grynkiewicz *et al.* [22]. Using wavelengths of 340 and 380 nm, Fura 2-AM was excited and the emitted light was analyzed at 520 nm.

Statistical analysis

For every treatment, sister cultures were used as controls in order to minimize the effect of variability in individual response. Data are shown as the mean \pm S.E.M for at least two independent experiments. Simple comparisons between two groups were made by Student's *t* test. Multiple comparisons were carried out by one-way analysis of variance (ANOVA) followed by Newman-Keuls Multiple Comparison Test. Differences were considered as statistically significant at $P < 0.05$.

Results

Stimulatory effect of ghrelin on $[Ca^{2+}]_i$ in cultured porcine somatotropes

Presence of somatotropes in pituitary cell cultures was confirmed by immunocytochemical staining with antibody raised against GH. GH immunoreactive cells comprised 40 % of the total pituitary cells in cultures. Increase in $[Ca^{2+}]_i$ above the baseline following hGHRH

application functionally identified GH cells. Perfusion with hGHRH (10 μ M) for 2 min increased $[Ca^{2+}]_i$ in somatotropes by 55 ± 3.0 nM (mean \pm SEM; $n = 232$; $P < 0.01$; Fig. 1A). Of the cells that responded to hGHRH, 98 % also responded to 1 μ M ghrelin applied 10 min after the application of hGHRH. Perfusion application of 1 μ M ghrelin for 2 min produced a prompt transient increase in $[Ca^{2+}]_i$ of 57 ± 3.0 nM ($n = 227$; $P < 0.01$; Fig. 1A), followed by the sustained decline to a plateau above the basal level. Similar calcium response was observed in previous work after treatment of isolated porcine somatotropes with nonpeptidyl GHS, L-692,585 (L-585) [23]. Experiments where reverse hemolytic plaque assay (RHPA) and $[Ca^{2+}]_i$ measurements were done concurrently demonstrated that calcium transient in cultured pituitary cells after application of L-585 coincided with GH secretion, implying that calcium transient evoked by ghrelin is also followed by GH release.

Plateau response was consistently observed after ghrelin application and was present even 30 min after application of ghrelin (Fig. 1A), suggesting that plateau was not result of incomplete washing of agonist or an artificial change in basal $[Ca^{2+}]_i$. When ghrelin was applied twice within 10 min, the second application of ghrelin, caused an increase in $[Ca^{2+}]_i$ in only 34 % of the cells that responded to the first application. The second application of 1 μ M ghrelin, 30 min after the first application increased $[Ca^{2+}]_i$ in 75 % of the cells that responded to the first application (Fig. 1A). A second application of ghrelin (1 μ M), during the plateau effect of the first application, did not have an additive effect on the increase in $[Ca^{2+}]_i$ (Fig. 1A). Application of the nonselective stimulus, potassium (50 mM), after hGHRH and ghrelin, resulted in elevated $[Ca^{2+}]_i$ by 93 ± 6.2 nM ($P < 0.01$) in all cells that responded to hGHRH and ghrelin (Fig. 1A).

The effect of ghrelin on calcium transient in cultured porcine somatotropes was dose dependent (Fig. 1B). In parallel run cultures, application of 500 nM ghrelin for 2 min elicited significantly bigger increase in $[Ca^{2+}]_i$ than 100 nM ghrelin (42 ± 3.1 nM, $n = 64$ vs 31 ± 2.0 nM, $n = 57$; $P < 0.05$), while after the application of 1 μ M ghrelin average rise of intracellular Ca^{2+} concentration (65 ± 3.4 nM; $n = 100$) was significantly bigger ($P < 0.01$) than response after application of 500 and 100 nM ghrelin.

To study possible interactions between GHRH and ghrelin, we determined responses of somatotropes to lower concentrations of ligands. Application of 5 μ M hGHRH increased $[Ca^{2+}]_i$ by 30.1 ± 1.7 nM ($n = 100$; $P < 0.01$; Fig. 2A) and subsequent application of 500 nM ghrelin increased $[Ca^{2+}]_i$ by 40 ± 3.1 ($n = 68$; $P < 0.01$; Fig. 2A). As shown in Fig 2B, simultaneous application of 5 μ M hGHRH and 500 nM ghrelin for 2 min produced a significant increase in $[Ca^{2+}]_i$ (35 ± 1.9 nM; $n = 110$; $P < 0.01$) but did not have an additive effect on the increase in $[Ca^{2+}]_i$.

Receptor mediation of stimulatory effect of ghrelin on $[Ca^{2+}]_i$

To determine whether the effect of ghrelin was receptor mediated, experiments were performed with (D-Lys³)-GHRP-6, a specific antagonist of GHS-R [6]. Perfusion of cultures with 100 μ M (D-Lys³)-GHRP-6 for 10 min before ghrelin, decreased $[Ca^{2+}]_i$ from 91 ± 4.8 nM to 64 ± 2.8 nM ($n = 101$; $P < 0.01$; Fig. 3B). In the presence of (D-Lys³)-GHRP-6, the increase in $[Ca^{2+}]_i$ evoked by ghrelin (1 μ M) was delayed for about 100 sec and decreased compared to response in controls (81 ± 4.7 , $n = 92$ vs 65 ± 4.0 nM, $n = 80$; $P < 0.05$; Fig. 3A,B). In control cultures 98 % of cells (92 of 94) that responded to hGHRH responded to ghrelin, while after pretreatment of cultures with (D-Lys³)-GHRP-6, only 79 % of cells (80 of 101) that responded to hGHRH responded to ghrelin. These results support the GHS-R mediating the effect of ghrelin increasing $[Ca^{2+}]_i$ in cultured porcine somatotropes.

Somatostatin (SRIH) decreases effect of ghrelin

Application of SRIH at concentrations of 10 μ M decreased intracellular calcium levels from 92 ± 3.5 nM to 69 ± 2.5 nM ($n = 115$; $P < 0.01$; Fig. 3C). After pretreatment of the cultures with 10 μ M SRIH for 10 min, 90 % of cells that responded to 10 μ M hGHRH responded to 1 μ M ghrelin (103 of 115). In parallel run controls, a similar percent of cells that responded to application of hGHRH responded to ghrelin (93 %). In the presence of 10 μ M SRIH, the increase in $[Ca^{2+}]_i$ evoked by perfusion application of 1 μ M ghrelin for 2 min was decreased (78 ± 4.1 nM, $n = 121$ vs 48 ± 2.7 nM, $n = 103$; $P < 0.01$; Fig. 3A,C).

Neuropeptide Y (NPY) decreases effect of ghrelin

NPY has a role in regulation of GH secretion [24, 25, 26] and thus the ability of ghrelin to evoke changes in $[Ca^{2+}]_i$ in the presence of NPY was examined. Treatment of somatotropes with NPY (1 μ M) for 10 min decreased $[Ca^{2+}]_i$ from 98 ± 2.4 nM to 77 ± 1.7 nM ($n = 144$; $P < 0.01$; Fig. 3D). In the presence of NPY, the increase in $[Ca^{2+}]_i$ evoked by 1 μ M ghrelin for 2 min was decreased compared to response in the control cultures (63 ± 3.5 nM, $n = 140$ vs 39 ± 2.1 nM, $n = 128$; $P < 0.01$; Fig. 3A,D). Thus NPY influenced the stimulatory effects of ghrelin.

Role of membrane depolarization and calcium channels in ghrelin effects

To investigate the requirement of extracellular calcium for hGHRH and L-585 induced calcium transients in cultured somatotropes, cells were bathed in calcium depleted HEPES with the addition of 1 mM EGTA to yield an extracellular free calcium level of 26 nM. It was necessary to have some calcium in the bathing medium, otherwise the cells detached from the culture substrate. The stimulatory effect of ghrelin was significantly decreased in calcium depleted saline (Fig. 4A,B). In low Ca^{2+} HEPES, only 42 % of the cells that responded to hGHRH also responded to ghrelin. The amplitude of ghrelin-evoked transient increase in $[Ca^{2+}]_i$ in low calcium HEPES was significantly smaller than in normal Ca^{2+} HEPES (65 ± 2.6 , $n = 200$ nM vs 29 ± 1.7 nM, $n = 76$; $P < 0.01$), and the second, sustained phase of calcium increase was abolished (Fig. 4B). These results suggest that extracellular calcium has an important role in the effect of ghrelin on GH secretory cells.

To investigate the contribution of voltage dependent Ca^{2+} channels in calcium influx evoked by ghrelin, experiments were performed in the presence of nifedipine, an antagonist of the L-type calcium channel. In control cultures, ghrelin (1 μ M) evoked a response in all cells ($n = 139$) that first responded to the hGHRH (10 μ M). In the presence of 100 nM nifedipine, 1 μ M ghrelin evoked a response in 90 % of the cells (64 of 71) that responded to hGHRH. The average increase in $[Ca^{2+}]_i$ was significantly smaller (60 ± 2.4 vs 39 ± 2.2 nM; $P < 0.01$; Fig. 4A,C) and the duration of the sustained phase of calcium increase was brief. Application of 50 mM K^+ , 10 min after ghrelin, resulted with significantly smaller increase in

$[Ca^{2+}]_i$ in the presence of nifedipine (112 ± 11.4 nM, $n = 139$ vs 48 ± 6.3 nM, $n = 71$; $P < 0.01$; Fig. 4A,C).

To determine the role of Na^+ in ghrelin-induced changes in $[Ca^{2+}]_i$, cultures were bathed in zero Na^+ solution (sodium was replaced with choline). In zero Na^+ HEPES, only 74 % of the somatotropes (88 of 119) responded to ghrelin (1 μ M) compared to 100% in normal HEPES solution. The amplitude of the increase in $[Ca^{2+}]_i$ evoked by ghrelin was smaller compared to that in normal sodium concentration (73 ± 7 nM vs 27 ± 2 nM, $P < 0.01$; Fig. 4A,D).

Signal transduction pathways, phospholipase C (PLC), and adenylyl cyclase - cyclic adenosine 3,5-monophosphate (AC-cAMP), are activated by ghrelin

Results with the application of ghrelin in depleted Ca^{2+} solution suggested that in ghrelin induced GH secretion, extracellular Ca^{2+} has an important role but that intracellular Ca^{2+} has some role, too. The involvement of intracellular Ca^{2+} stores ghrelin action was further investigated in experiments with U73122, a selective inhibitor of phospholipase C (PLC) [27]. Pretreatment of cultures for 10 min with 5 μ M U73122 significantly decreased the effect of 1 μ M ghrelin on calcium transient (65 ± 2.9 nM, $n = 82$ vs 44 ± 2.1 nM, $n = 73$; $P < 0.01$; Fig. 5A,B), implying an involvement of the PLC-IP₃ pathway in the ghrelin action.

To determine whether ghrelin activates cyclic adenosine 3,5-monophosphate pathway, cell cultures were pretreated with SQ-22536, an adenylyl cyclase inhibitor [28]. After 10 min application of SQ-22536 in concentrations 100 μ M, the amplitude of $[Ca^{2+}]_i$ increase evoked by 1 μ M ghrelin was significantly decreased (60 ± 2.4 , $n = 139$ vs 47 ± 1.8 , $n = 80$; $P < 0.01$; Fig. 5A,C), suggesting activation of AC by ghrelin.

Interactions between ghrelin and L-585 on calcium transient in porcine somatotropes: potential antagonism

Previously, we have demonstrated a stimulatory effect of L-585 on porcine somatotropes [23]. To investigate interaction between ghrelin, an endogenous GHS, and L-585, on calcium transients in cultured porcine somatotropes, these were applied in succession or simultaneously. The application of L-585 (10 μ M) for 2 min produced a prompt increase in

$[Ca^{2+}]_i$, followed by plateau phase (54 ± 3.5 ; $n = 97$; Fig. 6A). When L-585 (10 μ M) was applied 10 min after the application of 1 μ M ghrelin, it did not have an additive effect on $[Ca^{2+}]_i$ (33 ± 2.3 ; $n = 101$; $P < 0.01$; Fig. 6A,B). The effects of lower doses of ghrelin were examined. At 100 nM, ghrelin *per se* did not have influence on $[Ca^{2+}]_i$. In the presence of this concentration of ghrelin, applied 5 min before L-585, the average increase in $[Ca^{2+}]_i$ evoked by L-585 was greatly attenuated (39 ± 3.9 nM, $n = 34$ vs 24 ± 3.4 nM, $n = 19$; $P < 0.05$; Fig. 6C). In control cultures, all cells that responded to hGHRH also responded to L-585, whereas after pretreatment of cultures with 100 nM ghrelin only 27 % of somatotropes (19 of 70) responded to L-585. When ghrelin (1 μ M) was applied during the plateau phase of L-585-induced increase in $[Ca^{2+}]_i$ (10 min after 10 μ M L-585), the stimulatory effect of ghrelin was blocked and there was an unexpected decrease of $[Ca^{2+}]_i$ from 125 ± 2.9 nM to 108 ± 2.7 nM ($n = 80$; $P < 0.01$; Fig. 7A,B).

To determine whether the ghrelin response was blocked because it was applied during the plateau phase of L-585 action, we applied ghrelin after elevation of $[Ca^{2+}]_i$ in porcine somatotropes by 10 mM K^+ . Under such conditions the application of ghrelin resulted first with a prompt calcium increase that was followed by a sudden decrease in $[Ca^{2+}]_i$ (Fig. 7C).

Combined treatment with the two GHS, ghrelin and L-585 did not have an additive effect on $[Ca^{2+}]_i$. These data are summarized in Table 1. Applied simultaneously, 10 μ M L-585 and 1 μ M ghrelin produced an uncharacteristic brief increase in $[Ca^{2+}]_i$ without the typical prolonged plateau phase (Fig. 7D). The increase in $[Ca^{2+}]_i$ was not additive but, in fact, was lower ($P < 0.05$) than those evoked by either 10 μ M L-585 or 1 μ M ghrelin alone (Table 1). Similarly, simultaneous application of lower doses L-585 (5 μ M) and ghrelin (500 nM) did not have an additive effect on $[Ca^{2+}]_i$ (Table 1). Indeed in the presence of ghrelin, the L-585 evoked increase in $[Ca^{2+}]_i$ was less than observed with L-585 alone. These results suggest that ghrelin and L-585 may compete, probably at the receptor levels, with ghrelin acting as a partial antagonist.

Discussion

Studies in our laboratory have demonstrated that porcine somatotropes have a regulated secretory pathway in which secretory vesicles fuse with the plasma membrane in response to stimulus such as GHRH or L-585 [29]. In the present study, we have shown that ghrelin has a direct, dose dependent, stimulatory effect on $[Ca^{2+}]_i$ in isolated porcine somatotropes. It is presumed that this increase in $[Ca^{2+}]_i$ evoked by ghrelin is followed by GH release [30]. Secretion of GH from perfused rat anterior pituitary cells reached a maximum 2 min after ghrelin application [31]. This coincides temporally with peak increase in $[Ca^{2+}]_i$ evoked by ghrelin in our experiments, suggesting calcium involvement in the GH release evoked by ghrelin. Similarly, in previous experiments on isolated porcine somatotropes with simultaneous measurements of GH release and calcium transients we have shown that L-585, a nonpeptidyl GHS, evoked an increase in $[Ca^{2+}]_i$ that coincided with GH release [23]. An increase in $[Ca^{2+}]_i$ was observed with ghrelin in all cells that responded to hGHRH. It may be questioned whether these act via the same signal transduction pathway.

The amplitude of increase in $[Ca^{2+}]_i$ evoked by ghrelin was similar to that with hGHRH with the latter at the 10-fold greater concentration. This may suggest that ghrelin is more potent than GHRH in stimulating rapid release of GH. In human studies, it is shown that the potency of ghrelin for GH release is similar to those of GHS, but greater than GHRH, indicating a distinctive pharmacological action [15, 16]. However, in rat anterior pituitary cells ghrelin caused weaker GH secretion than GHRH [31], suggesting that potency of ghrelin is species-dependent. There was no evidence for any heterologous desensitization between hGHRH and ghrelin. However, homologous desensitization was observed with repeated application of ghrelin. Repeated application of ghrelin after 10 and 30 min evoked a response in 34% and 75% of cells that responded to the first application. Previous studies have shown that there is no cross-desensitization between GHRH and GHS in GH release, while homologous desensitization does occur [2, 32]. Release of GH from dispersed rat pituitary cells was decreased after serial ghrelin stimulation at 1 h intervals, also indicating that it strongly desensitized somatotropes [31].

At low doses ghrelin synergizes with GHRH on GH secretion from the human pituitary gland. This also suggests different sites of action for these secretagogues [16, 17].

Simultaneous application of hGHRH and ghrelin in the present study, however, did not have an additive effect on intracellular calcium concentration in cultured porcine somatotropes. In agreement with our results, stimulation with ghrelin and GHRH did not elicit either a synergistic or an additive GH response from the dispersed rat pituitary cells [31], suggesting that ghrelin indirectly affects GH release from pituitary gland via hypothalamic mediators. In previous study [23] simultaneous application of GHRH and L-585 also did not have an additive effect on calcium increase in porcine somatotropes, implying that hypothalamus is important site of action of both endogenous and exogenous GHS and that for their maximal effect an intact hypothalamo-pituitary axis is necessary.

To investigate whether the stimulatory effect of ghrelin on $[Ca^{2+}]_i$ in porcine somatotropes is mediated by the GHS-R, we used, a putative competitive GHS-R antagonist, (D-Lys³)-GHRP-6 [6]. The stimulatory effect of ghrelin in the presence of used (D-Lys³)-GHRP-6 was considerably decreased. This supports the mediation by GHS-R of ghrelin's effect on calcium transient in porcine somatotropes. While (D-Lys³)-GHRP-6 failed to completely depress the increase in $[Ca^{2+}]_i$ evoked by ghrelin, this can be explained by a lower affinity of (D-Lys³)-GHRP-6 for GHS-R compared to that of ghrelin [9, 33]. In CHO-GHSR62 cells, in the presence of 10^{-4} M (D-Lys³)-GHRP-6, $[Ca^{2+}]_i$ increase evoked by lower doses of ghrelin was depressed, but not in the presence of a high dose of ghrelin, indicating that ghrelin is competitively inhibited by the antagonist [9]. It is also possible that several subtypes of GHS-R exist [34, 35].

Somatostatin decreased, but did not abolish the effect of ghrelin on calcium transient in porcine somatotropes. In our previous study with L-585, SRIH was also more effective in suppressing the stimulatory effect of hGHRH compared to L-585 [23]. SRIH alone decreased $[Ca^{2+}]_i$. The effects of SRIH on somatotropes are mediated either by inhibiting of cAMP formation [36] or via decreased Ca^{2+} influx as a result of an increase in K^+ conductance and hyperpolarization of somatotropes [37]. GHSs, and probably ghrelin, can behave as functional SRIH antagonists by inhibiting K^+ channels and depolarizing the plasma membrane of the somatotropes [38].

It has been suggested that NPY suppresses the release of GH by reciprocal regulation of GHRH and SRIH neurons [39]. Indeed, centrally administered NPY inhibited GH

secretion in rats [24, 26] and pigs [41]. In the present study, pretreatment of cultured porcine pituitary cells with NPY decreased the effect of ghrelin on $[Ca^{2+}]_i$. Direct inhibitory effects of NPY on GH secretion have been observed with human pituitary somatotrophic tumours in cell culture with reductions of GHRH stimulatory effects by NPY [25]. Our results, supported by those with human tumor cells, clearly demonstrate that NPY can inhibit GH secretion also by suppressing the ghrelin stimulatory effect on the somatotrope directly. In addition, NPY decreased basal $[Ca^{2+}]_i$. It is possible that NPY, similar to the situation with SRIH, exerts its inhibitory effect on ghrelin by modulating $[Ca^{2+}]_i$.

Depletion of extracellular calcium greatly diminished but did not completely abolish the stimulatory effect of ghrelin on porcine somatotropes. The latter suggests that while ghrelin does mobilize Ca^{2+} from intracellular stores, Ca^{2+} influx has the major contribution to calcium transient. In somatotropes, the major Ca^{2+} channels are the voltage-gated T- and L-types [41]. Perfusion of porcine cells with nifedipine decreased the effect of ghrelin. This indicates an involvement of L-type Ca^{2+} channels in calcium influx induced by ghrelin, and that influx of calcium is a crucial step in the action of ghrelin, as in the action of L-585 [23].

The stimulatory effect of ghrelin was decreased in a zero Na^+ environment, suggesting that ghrelin can depolarize somatotropes at least, partially, through sodium channels. Potassium channels may also be involved in the action of ghrelin, because activation of GHS-R leads to inhibition of K^+ channels, allowing the entry of Ca^{2+} through voltage-gated channels [2, 38]. In addition, different second messengers can activate calcium channels. Cyclic adenosine monophosphate (cAMP), protein kinase A and C, and phospholipase C are possible signaling systems involved in the action of GHS [34, 38], and are likely involved in the ghrelin action. In our experiments U73122, a PLC inhibitor, decreased the effect of ghrelin on calcium transient, indicating the involvement of PLC/ IP_3 pathway in ghrelin action on porcine somatotropes. Pretreatment of porcine somatotropes with SQ-22536, an adenylyl cyclase inhibitor, also decreased the stimulatory effect of ghrelin on calcium transient, implying that the binding of ghrelin to GHS-R activates adenylyl cyclase. Our results indicated that the first phase of ghrelin-evoked calcium increase resulted from intracellular Ca^{2+} mobilization, whereas the second phase represents calcium influx. In low calcium HEPES or with nifedipine or in zero sodium HEPES, this second sustained phase was almost

completely abolished. Very similar results and conclusions were drawn from studies examining the ability of L-585 to affect $[Ca^{2+}]_i$ in low calcium HEPES or with nifedipine or in zero sodium HEPES in porcine somatotropes [23]. This suggested that ghrelin and L-585 possess similar mechanisms of action. The commonality in action between ghrelin and peptidyl GHS has been previously shown. The endocrine responses to ghrelin were not modified by the coadministration of hexarelin [16]. After repeated administration of ghrelin or GHRP-2, desensitization of the GH response was induced by the homologous agent, and also by the heterologous agent in crossover studies with the two peptides [18].

In the present study, we have for the first time investigated interactions between ghrelin and the nonpeptidyl GHS, L-585. To examine interactions between ghrelin and L-585, we either applied these agonists in succession or simultaneously. L-585 had no additive effect on $[Ca^{2+}]_i$ when applied 10 min after ghrelin (that is during the plateau increase in $[Ca^{2+}]_i$). Moreover, the response evoked by L-585 was almost completely abolished when applied in the presence of 100 nM ghrelin; a concentration that alone did not have any effect on $[Ca^{2+}]_i$. The stimulatory effect of ghrelin was blocked when it was applied during the plateau phase of L-585 action, and instead of an increase, an abrupt decrease in $[Ca^{2+}]_i$ occurred. Similar decrease in $[Ca^{2+}]_i$ was detected when ghrelin was applied during the plateau increase induced by 10 mM K^+ .

Simultaneous application of ghrelin and L-585 did not have additive effect on calcium transient. Indeed the change in $[Ca^{2+}]_i$ was reduced compared to either GHS alone. Together our experiments with ghrelin and L-585 indicate antagonistic effects between these two agonists. Competition between ghrelin and L-585 probably occurs at the GHS-R on somatotropes, which may involve binding to the different but overlapping domains on the same receptor. Alternatively, competition between these agonists may occur downstream from the GHS-R or sub-types.

In summary, ghrelin has a direct stimulatory effect on porcine somatotropes. During the first phase of the ghrelin-induced calcium transient activation of different signal transduction pathways result with calcium mobilization from internal stores, whereas a second prolonged phase results from calcium influx as a consequence of somatotrope depolarization by ghrelin acting on Na^+ and K^+ channels and the activation of calcium

channels by acting on different second messengers. Similar responses were previously obtained with L-585 suggesting a similar mechanism of action between ghrelin and nonpeptidyl GHS. Unexpectedly, there were antagonistic effects of ghrelin and L-585 on calcium transients in porcine somatotropes. The basis for these is unknown but they do not appear to represent simple competitive antagonism.

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Table 1. Effect of ghrelin and L-585, alone and together, on changes in $[Ca^{2+}]_i$ in pig somatotropes.

Treatment	Concentration (μ M)	Increase in $[Ca^{2+}]_i$ (nM) \pm S.E.M. (n=)
Ghrelin	0.5	34 ± 7.0 (26) ^{ab}
L-585	5	49 ± 5.5 (44) ^a
Ghrelin + L-585	0.5 + 5	29 ± 5.0 (31) ^b
Ghrelin	1	62 ± 8.4 (22) ^a
L-585	10	65 ± 8.1 (22) ^a
Ghrelin + L-585	1 + 10	32 ± 3.5 (32) ^b

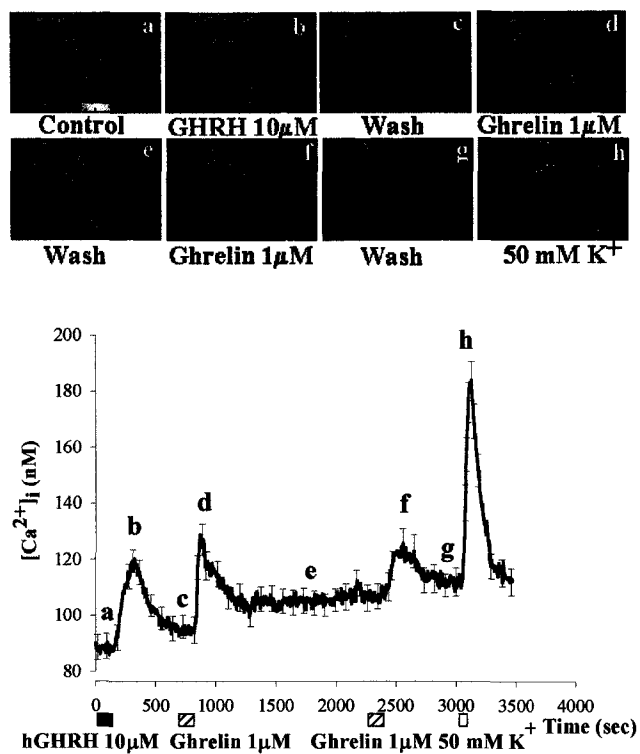
Different superscript numbers indicate difference, $P < 0.05$

Figure 1. Stimulatory effect of ghrelin on $[Ca^{2+}]_i$ in porcine somatotropes:

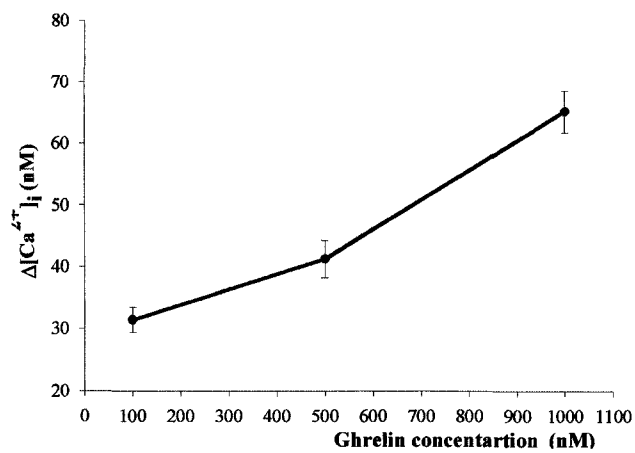
A) The sequence of pseudocolor images and kinetics of the $[Ca^{2+}]_i$ changes illustrate response of isolated porcine pituitary cells to application of 10 μ M hGHRH, two consecutive applications of 1 μ M ghrelin, and 50 mM K^+ ($n = 22$). Somatotropes were functional identified by 2 min application of 10 μ M hGHRH. Subsequent administration of 1 μ M ghrelin evoked increase in $[Ca^{2+}]_i$ in 98% of somatotropes. The repeated administration of ghrelin after 30 min did not have additive effect on the $[Ca^{2+}]_i$ increase, suggesting existence of homologous desensitization of GHS receptors. Application of 50 mM K^+ increased $[Ca^{2+}]_i$ in all cells that responded to hGHRH and ghrelin. Panels of images of calcium transient were taken at the time indicated on time course histogram.

B) Application of ghrelin in concentrations of 100 nM, 500 nM and 1 μ M, dose-dependently increased $[Ca^{2+}]_i$ in isolated porcine somatotropes. The application of 500 nM ghrelin for 2 min evoked a smaller response than application of 1 μ M ghrelin in cultures run in parallel ($P < 0.01$), while the application of 100 nM ghrelin for 2 min evoked a smaller response than application of 500 nM ghrelin in cultures run in parallel ($P < 0.05$).

A



B



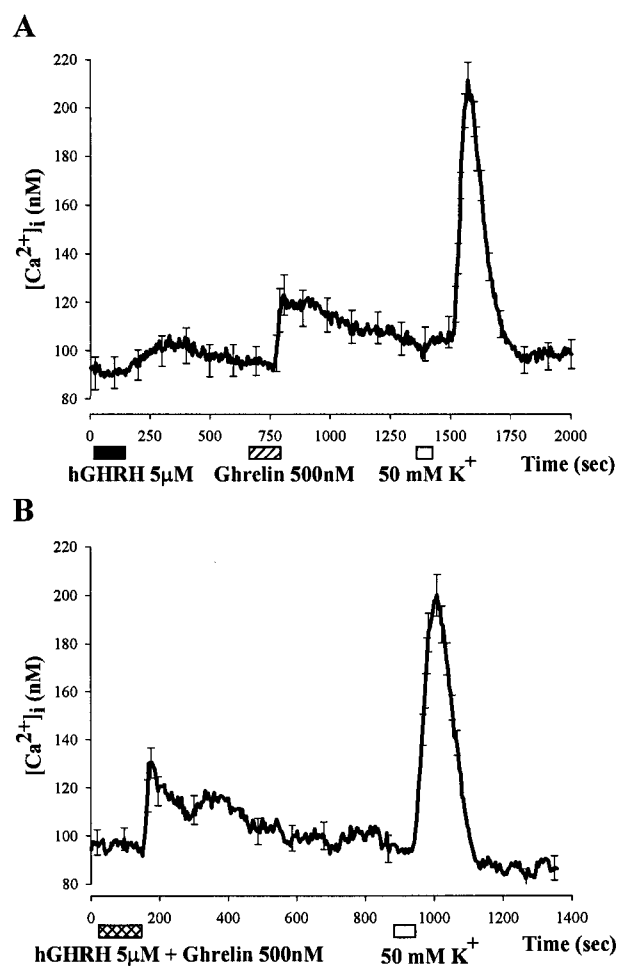


Figure 2. Simultaneous application of hGHRH and ghrelin did not have an additive effect on calcium transient:

A) Increase in $[Ca^{2+}]_i$ after 2 min application of hGHRH (5 μ M) and ghrelin (500 nM) ($n = 21$)

B) Increase in $[Ca^{2+}]_i$ after simultaneous application of hGHRH (5 μ M) and ghrelin (500 nM) for 2 min, was not significantly different from response elicited by either agonist alone ($n = 28$).

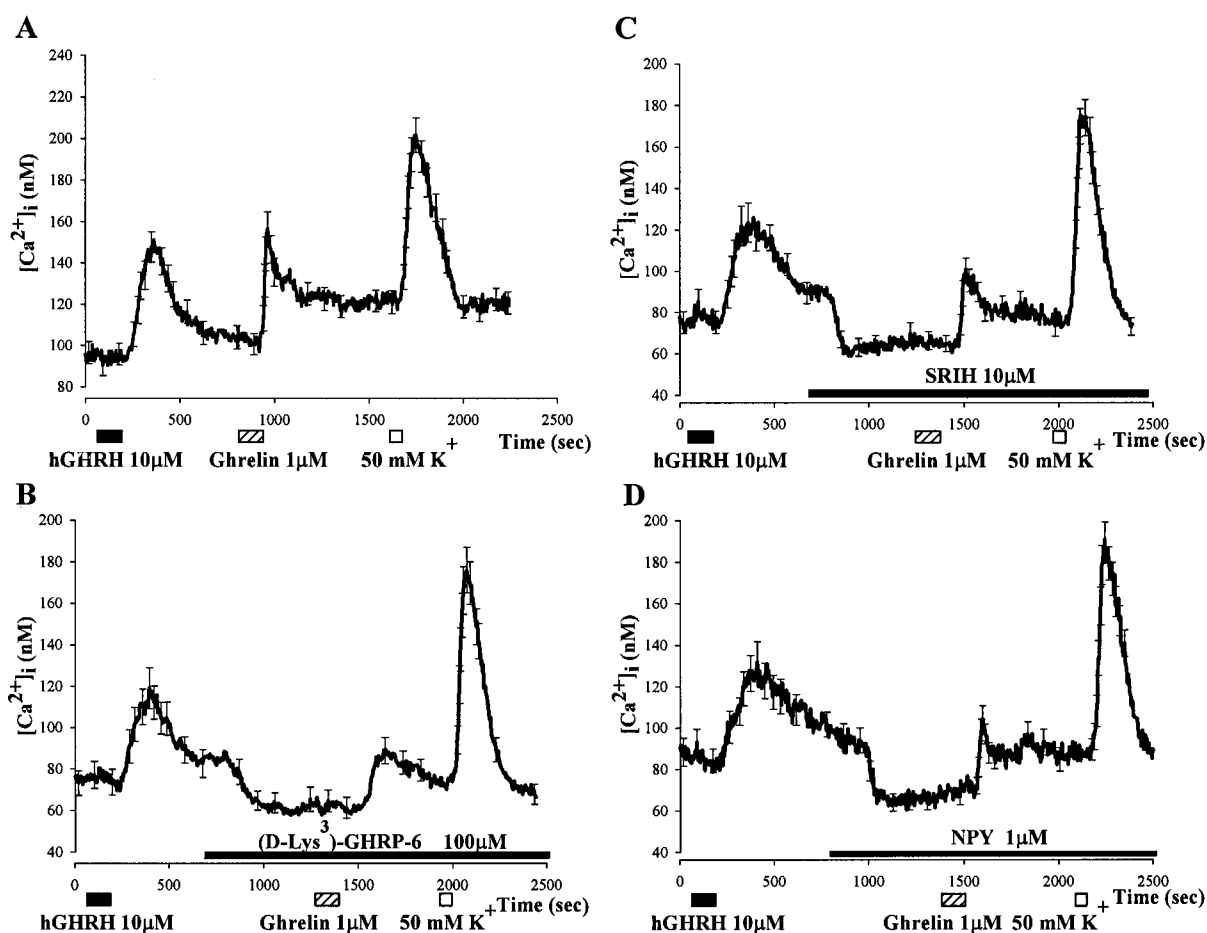


Figure 3. Inhibitory effects of (D-Lys³)-GHRP-6, SRIH and NPY on calcium transient evoked by 2 min application of 1 μ M ghrelin:

A) Increase in $[Ca^{2+}]_i$ after sequential application of hGHRH (10 μ M), ghrelin (1 μ M) and 50 mM K^+ ($n = 27$).

B) Pretreatment of cultures for 10 min with (D-Lys³)-GHRP-6 (100 μ M) decreased $[Ca^{2+}]_i$, and attenuated the response evoked by ghrelin (1 μ M) ($n = 42$; $P < 0.05$), suggesting that stimulatory effect of ghrelin is mediated through GHS-R.

C) Application of SRIH (10 μ M) for 10 min decreased $[Ca^{2+}]_i$ and reduced the response to ghrelin (1 μ M) ($n = 37$; $P < 0.01$).

D) Pretreatment of cultures for 10 min with NPY (1 μ M) decreased both $[Ca^{2+}]_i$ and the increase in $[Ca^{2+}]_i$ evoked by ghrelin (1 μ M) ($n = 21$; $P < 0.01$).

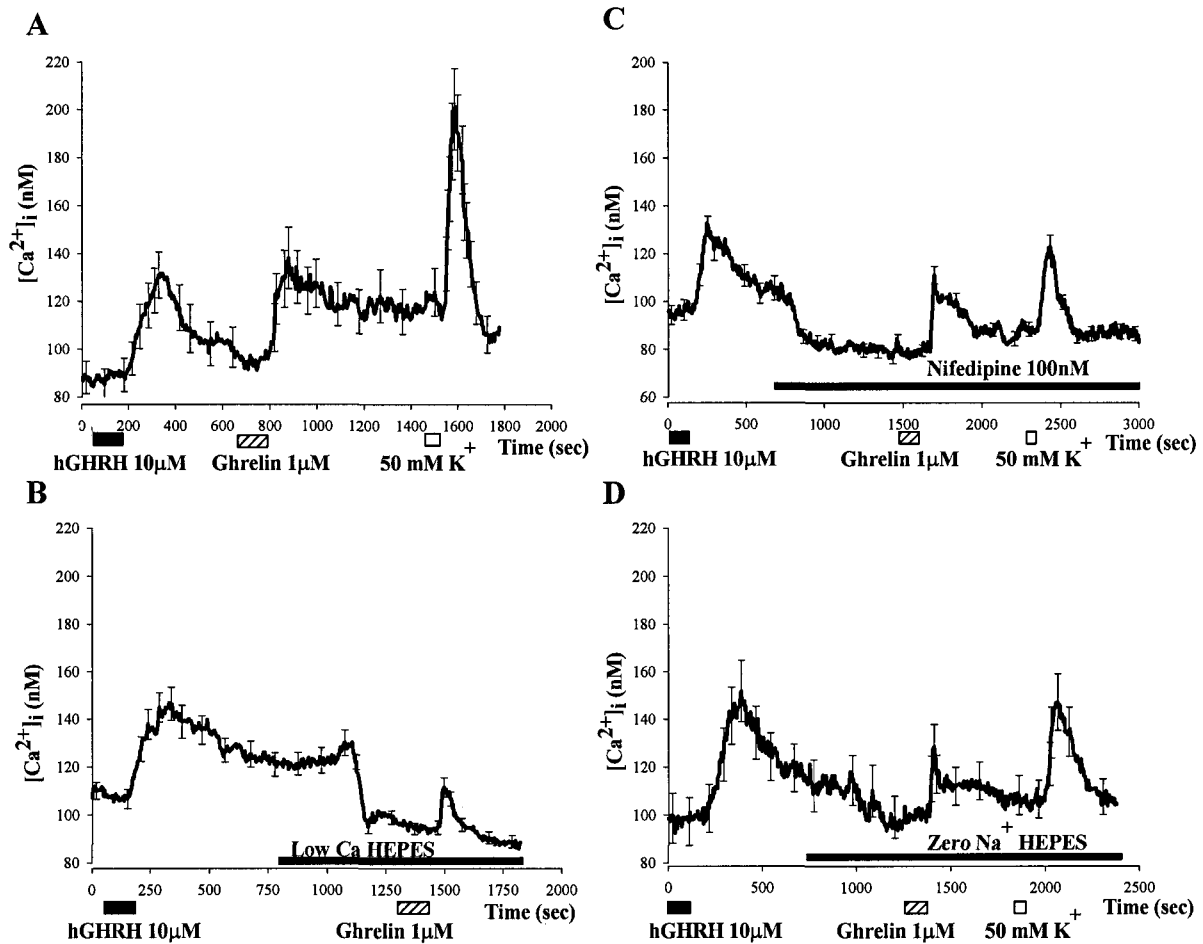


Figure 4. Calcium and sodium channels are involved in ghrelin induced calcium transient in isolated porcine somatotropes:

A) Effects of hGHRH (10 μ M), ghrelin (1 μ M), and 50 mM K^+ on calcium transient in isolated porcine somatotropes ($n = 26$).

B) Bathing the cultures in low Ca^{2+} solution decreased baseline $[Ca^{2+}]_i$ and dramatically reduced the stimulatory effect of ghrelin (1 μ M) on $[Ca^{2+}]_i$ in isolated porcine somatotropes ($n = 22$; $P < 0.01$).

C) Perfusion of culture with nifedipine (100 nM), a blocker of L-type Ca channels, decreased stimulatory effect of ghrelin (1 μ M) ($P < 0.05$) and 50 mM K^+ on isolated porcine somatotropes ($n = 19$; $P < 0.01$).

D) Removing of Na^+ ions from the bathing solution decreased the effect of 1 μ M ghrelin on calcium transient ($n = 18$; $P < 0.01$).

Figure 5. PLC-IP₃ and AC-cAMP pathways are involved in stimulatory effect of ghrelin on cultured porcine somatotropes:

A) Control effect of 10 μ M hGHRH, 1 μ M ghrelin, and 50 mM K⁺ on calcium transient in isolated porcine somatotropes ($n = 33$).

B) Pretreatment of the cultured porcine pituitary cells with 5 μ M U73122 decreased the stimulatory effect of ghrelin ($P < 0.01$; $n = 24$).

C) in the presence of 100 μ M SQ-22536 (SQ), adenylyl cyclase inhibitor, stimulatory effect of ghrelin was decreased ($P < 0.01$; $n = 25$).

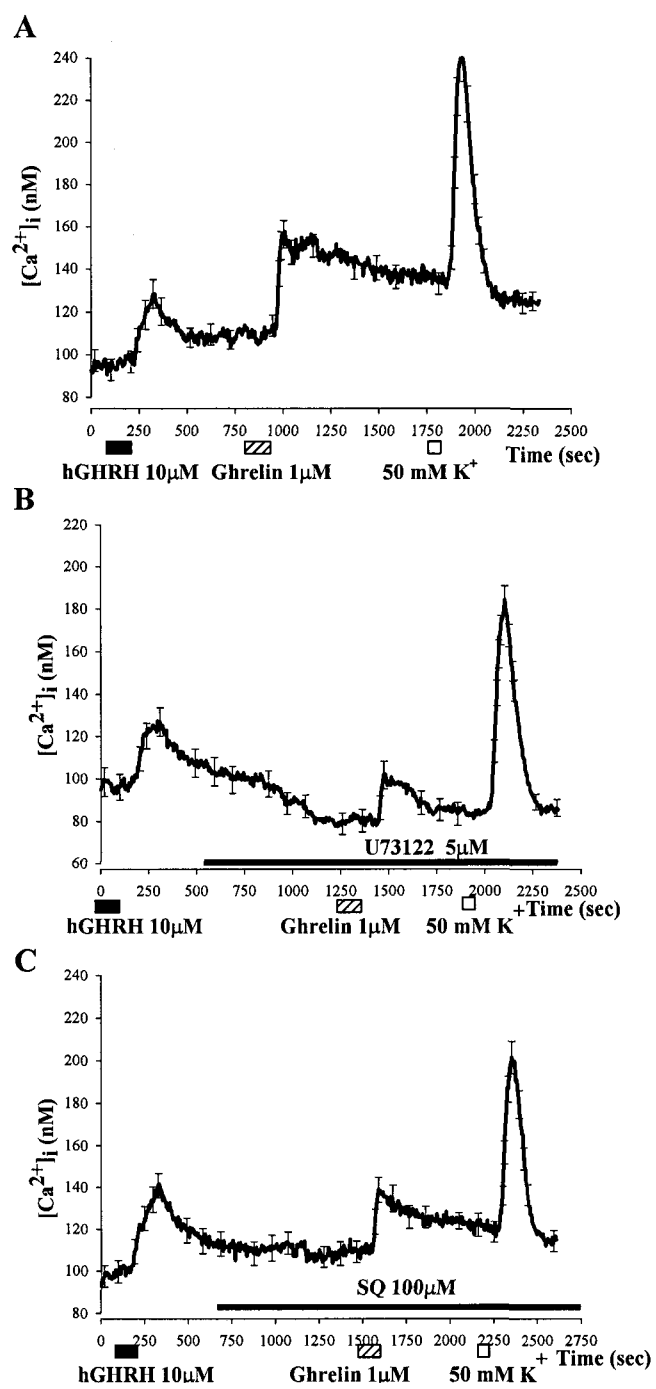


Figure 6. Inhibitory effects of ghrelin on calcium transients evoked by L-585 in isolated porcine somatotropes:

A) Control studies on calcium transients in isolated porcine somatotropes evoked by 2 min application of hGHRH (10 μ M) and L-585 (10 μ M) ($n = 29$).

B) Application of L-585 (10 μ M), 10 min after application of ghrelin (1 μ M), did not have an additive effect on $[Ca^{2+}]_i$ in isolated porcine somatotropes ($n = 58$).

C) Ghrelin (100 nM), alone did not affect $[Ca^{2+}]_i$, but the changes in $[Ca^{2+}]_i$ evoked by 10 μ M L-585 was almost completely blocked ($n = 7$).

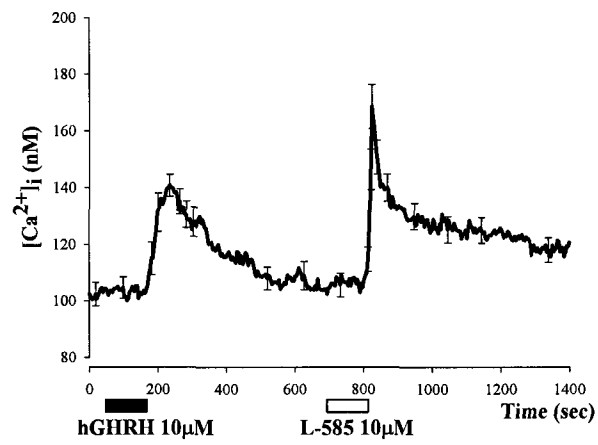
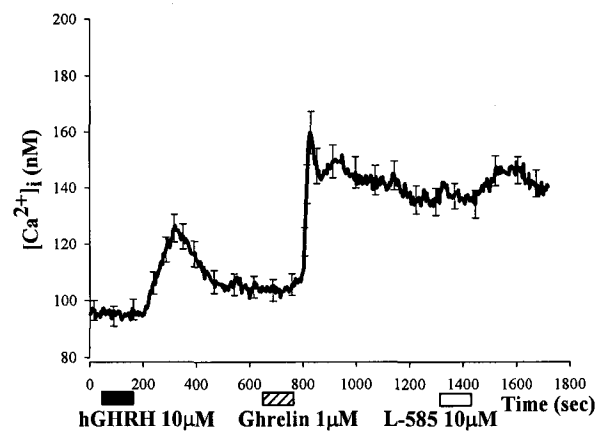
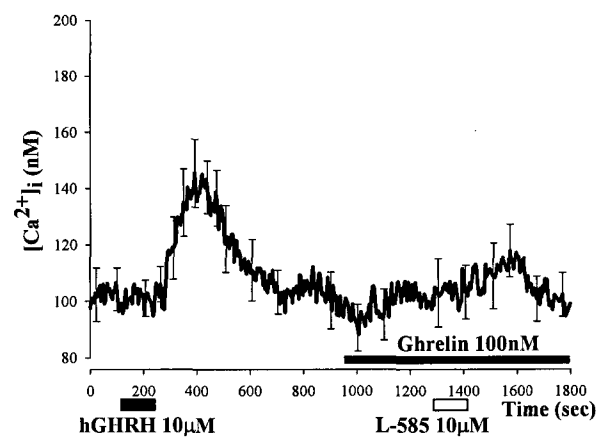
A**B****C**

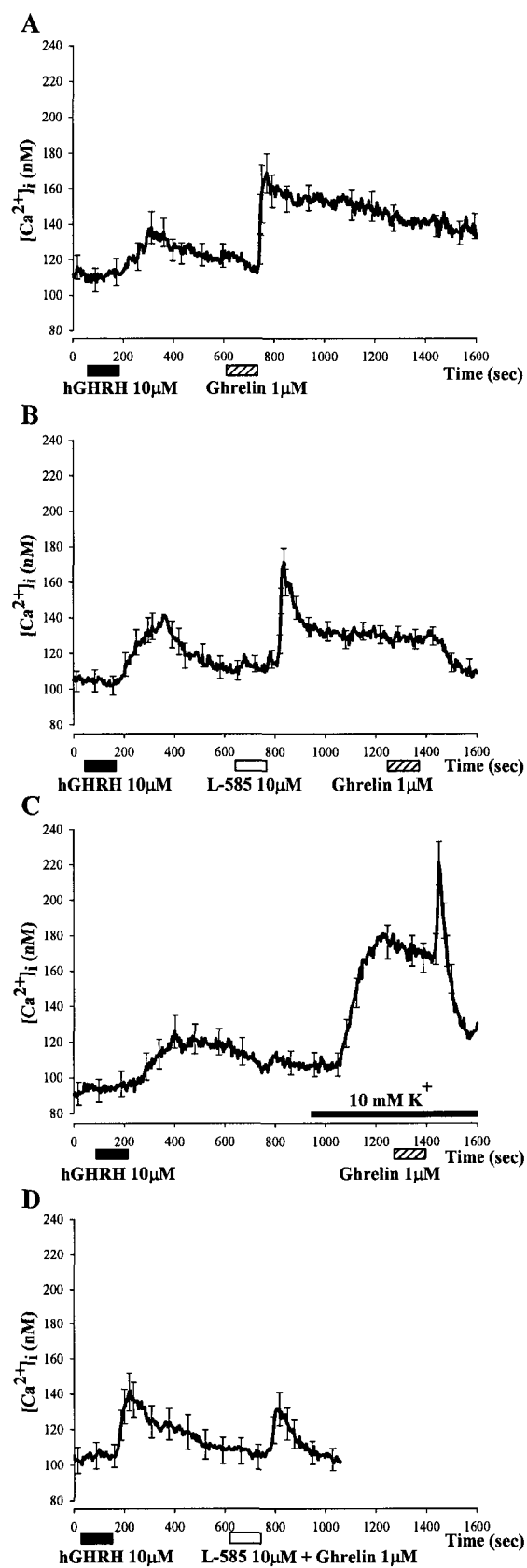
Figure 7. Inhibitory effect of L-585 on calcium transient evoked by ghrelin in isolated porcine somatotropes:

A) Control effect of 10 μ M hGHRH and 1 μ M ghrelin on calcium transient in isolated porcine somatotropes ($n = 20$).

B) Stimulatory effect of 1 μ M ghrelin on calcium transient in isolated porcine somatotropes was blocked when ghrelin was applied during the plateau phase of calcium increase evoked by L-585. Instead of increase in $[Ca^{2+}]_i$, application of ghrelin produced sudden drop in $[Ca^{2+}]_i$ ($n = 21$).

C) Application of 1 μ M ghrelin for 2 min during the plateau phase of $[Ca^{2+}]_i$ increase evoked by perfusion application of 10 mM K^+ produced prompt increase in $[Ca^{2+}]_i$ followed by sudden drop in $[Ca^{2+}]_i$ ($n = 46$).

D) Simultaneous application of 10 μ M L-585 and 1 μ M ghrelin for 2 min produced a uncharacteristic, brief and not additive effect on the $[Ca^{2+}]_i$ increase ($n = 26$).



CHAPTER 5. GENERAL CONCLUSIONS

This dissertation presents the results of investigation the sites and cellular mechanisms of action of an exogenous, nonpeptidyl GH secretagogue - L-585, and endogenous GH secretagogue - ghrelin, on cultured porcine somatotropes. Understanding of the role of GHS in hypothalamic-pituitary axis has been increasing in recent years. This research contributes to this body of knowledge by showing that both, exogenous and endogenous GHS have direct stimulatory effect on $[Ca^{2+}]_i$ in cultured porcine somatotropes, that is followed by release of GH. These results strongly suggest that GHS are indeed physiological regulator of GH secretion.

General summary

In the first study, the cellular mechanism of action of L-585 on isolated porcine somatotropes was investigated. ICC using antibody against GH confirmed the presence of somatotropes in porcine pituitary cultures. The ability of L-585 to stimulate Ca^{2+} transients and GH secretion from cultured porcine somatotropes was tested. All cells, that were identified as somatotropes by increase in $[Ca^{2+}]_i$ after hGHRH application, responded also to perfusion application of L-585. Application of 10 μ M L-585 for 2 min evoked prompt transient in $[Ca^{2+}]_i$, followed by sustained decline to a plateau above the basal level. In experiments where RHPA and $[Ca^{2+}]_i$ measurements were done concurrently, it was shown that increase in $[Ca^{2+}]_i$ evoked by L-585 coincides with GH secretion. Incubation of cultured cells with L-585 for 3 h resulted with increased number and size of plaques around somatotropes, showing stimulatory effect of L-585 on GH secretion. Simultaneous application of L-585 and hGHRH did not have an additive effect on $[Ca^{2+}]_i$. hGHRH and L-585 exert their stimulatory effect on $[Ca^{2+}]_i$ in porcine somatotropes through different receptors, since SP analogue and GHRH receptor antagonist, influenced just effect of hGHRH. The depletion of calcium from bathing medium and blocking of L-calcium channels by nifedipine decreased initial peak increase and abolished the plateau component of L-585-induced calcium changes, suggesting involvement of extracellular calcium. Both adenylyl cyclase (AC) inhibitor (SQ-22536), and PLC inhibitor (U73122), significantly decreased

stimulatory effect of L-585, implying that L-585 mobilizes Ca^{2+} from internal stores by the activation of AC-cAMP and PLC- inositol 1,4,5-trisphosphate pathways (IP_3). These results together suggest that rapid increase in $[\text{Ca}^{2+}]_i$ induced by L-585 originated from the intracellular stores, while the plateau phase resulted from somatotropes depolarization and extracellular calcium entry.

In the second study plasma membrane structures involved in GH secretion from isolated porcine somatotropes were observed using AFM. Recently, new group of plasma membrane structures, named 'pits' and 'depressions', were found on apical membrane of pancreatic acinar cells. It was demonstrated that structures are sites at plasma membrane where vesicles dock and fuse to release content. In this study, the same structures were observed at the plasma membrane of somatotropes. On the plasma membrane of resting somatotropes were found 'pits' containing several 'depressions', or fusion pores, with diameter of 100-200 nm. After stimulation of cultured porcine somatotropes with nonpeptidyl GHS – L-585, enlargement of 'depressions' for about 40 % was observed. When cells stimulated with L-585 were fixed and later exposed to 30 nm gold-tagged GH-antibody, fusion pores were lost due to large amounts of gold-tagged antibody binding at these sites. These results together have shown that 'depressions' are structures at plasma membrane of somatotropes involved in hormone secretion. The existence of 'pits' and 'depressions' both in exocrine and neuroendocrine cells imply that these structures may be common for all secretory cells.

The effect of ghrelin, an endogenous ligand for GHS-R, on intracellular Ca^{2+} concentration in isolated porcine somatotropes was investigated in the third study using a calcium imaging system. Somatotropes were functionally identified by application of hGHRH. Subsequent application of 1 μM ghrelin increased $[\text{Ca}^{2+}]_i$ in 98 % of the cells that responded to hGHRH. Repeated application of 1 μM ghrelin after 10 and 30-min increased $[\text{Ca}^{2+}]_i$ in 34% and 75% of cells that responded to the first application, suggesting that homologue desensitization of GHS-R occurred. Effect of ghrelin on somatotropes was dose dependent. Ghrelin in concentration of 100 and 500 produced significantly smaller response than 1 μM ghrelin. Similar to the results observed with synthetic GHS, simultaneous application of ghrelin and hGHRH did not cause an additive increase in $[\text{Ca}^{2+}]_i$. The

stimulatory effect of ghrelin on somatotropes was receptor mediated and it was decreased in the presence of (D-Lys³)-GHRP-6, a specific receptor antagonist of GHS-R. Ghrelin-induced increase of $[Ca^{2+}]_i$ was reduced in the presence of SS and NPY. The experiments in low calcium saline and with L-calcium channel blocker - nifedipine have shown that influx of calcium through calcium voltage dependent channels is involved in calcium increase evoked by ghrelin. The sodium channels are also involved in ghrelin-induced calcium a transient as was shown by experiments in a zero Na⁺ solution. Pretreatment of cultures with adenylyl cyclase (AC) inhibitor (SQ-22536), and PLC inhibitor (U73122), significantly decreased stimulatory effect of ghrelin, implying that activation of AC-cAMP and PLC- inositol 1,4,5-trisphosphate pathways (IP₃) were involved in ghrelin evoked calcium mobilization from internal stores. These results together suggested that rapid increase in $[Ca^{2+}]_i$ induced by L-585 originated from the intracellular stores, while the plateau phase resulted from somatotropes depolarization and extracellular calcium entry. The application of nonpeptidyl GHS, L-585 resulted with changes in $[Ca^{2+}]_i$ similar to those induced by ghrelin. When L-585 was applied 10 min after the application of ghrelin, it did not cause an additive increase in $[Ca^{2+}]_i$. Pre-application of the low dose of ghrelin, that alone did not influence the $[Ca^{2+}]_i$, greatly attenuated the stimulatory effect of subsequently applied L-585. Application of ghrelin, 10 min after the application of L-585, resulted with an unexpected decrease in $[Ca^{2+}]_i$. Simultaneous application of ghrelin and L-585 did not have an additive effect on $[Ca^{2+}]_i$ increase, but resulted with reduced change in $[Ca^{2+}]_i$ compared to either GHS alone. Our results suggest that the action of ghrelin and synthetic GHS closely parallel each other, and that antagonistic effects between these two agonists may result from competition at the receptor level and/or downstream from GHS-R.

General discussion

The central regulation of pituitary GH release is mediated by opposing actions of two hypothalamic hormones, GHRH and SS (Frohman *et al.* 1992, Muller *et al.* 1999). In addition, synthetic GHS exhibit strong GH-releasing activity by acting both on the pituitary and hypothalamus, where GHS-R are present (Dickson *et al.* 1995, Smith *et al.* 1996, Casanueva & Dieguez 1999). In fact, an endogenous ligand for GHS-R, ghrelin, was

discovered and specifically stimulated GH secretion both *in vitro* and *in vivo* (Kojima *et al.* 1999). Thus, ghrelin is thought to be the third physiological regulator of GH secretion. Although the effects of various non-peptidyl GHS on GH secretion in pigs have been analyzed in several studies *in vivo* (Chang *et al.* 1995, 1996, Hickey *et al.* 1996), no data are yet available on the *in vitro* action of those compounds in swine. Analysis of the direct effects of GHS on porcine somatotropes appears as a required step to better understand the possible role of these secretagogues on the regulation of GH in this species. The knowledge of the mechanisms by which ghrelin and other specific GHS stimulate GH secretion may ultimately be applied to production of high quality meat. Pork is an important U.S. agricultural commodity and a major protein source worldwide. Administration of ghrelin has been shown to stimulate pituitary GH secretion, but also increase body growth appetite and fat deposition (Wang *et al.* 2002). The optimization of GH secretion in pigs would be expected to increase growth rate and improve the quality of the meat by reducing adipose tissue. This further would improve human nutrition and reduce consumption of fat, particularly saturated fat – a cause for concern relative to cardiovascular disease and obesity in the US population. One primary objective of this thesis is to increase our understanding of cellular mechanisms of action of GHS in pigs. In order to ascertain the direct actions of GHS and ghrelin on porcine somatotropes we have evaluated their effects on GH release and intracellular calcium concentration from cultured pituitary cells.

Rapid growth in the young pigs requires an episodic pattern of GH secretion. In *in vivo* experiments of Hickey *et al.* (1996), L-585 had direct stimulatory effect on the pituitary gland, but an intact stalk connecting the pituitary and hypothalamus was necessary for maximal GH response. The studies included in this thesis focused on determining the mechanism of GHS action at the pituitary level. Perfusion application of L-585 had direct stimulatory effect on $[Ca^{2+}]_i$ in cultured porcine somatotropes. From this study one of the most important findings was an increase in $[Ca^{2+}]_i$ in cultured somatotropes evoked by L-585, coincides with GH release. The experiments with concurrent measurements of calcium transient and RHPA have demonstrated that hemolytic plaques were formed only around cells that responded with increase in $[Ca^{2+}]_i$ to L-585 application, confirming that those cells release GH. The stimulatory effect of L-585 on GH secretion from cultured porcine

somatotropes was quantified with RHPA. Size and number of plaques were increased in somatotropes exposed to L-585 comparing to cells in the resting state, showing that GH release from somatotropes is increased after exposure to L-585. In calcium imaging experiments it was demonstrated that increase in $[Ca^{2+}]_i$ evoked by L-585 application is greater than after application hGHRH, implying that L-585 is more potent than hGHRH in the rapid release of GH. *In vivo* experiments in pigs have shown that L-585 is indeed more efficacious than GHRH in releasing circulating GH (Hickey *et al.* 1996).

It is well documented that GHS and GHRH act through different receptors on the somatotropes plasma membrane (Wu *et al.* 1996). In cultured porcine somatotropes stimulatory effects of hGHRH and L-585 on $[Ca^{2+}]_i$ were also mediated through different receptors. When hGHRH and L-585 were applied in succession, there was no heterologous desensitization, while homologous desensitization occurred after repeated application of ligands. In addition, SP analogue blocked and GHRH receptor antagonist decreased the stimulatory effect of hGHRH, while these treatments did not have influence on the calcium transient evoked by L-585. *In vivo* experiments with pigs have shown that L-585 synergies with GHRH (Hickey *et al.* 1996), but in the present *in vitro* study hGHRH and L-585 exhibited no additive effect on intracellular calcium concentration. This is in correlation with previous studies where GHS also exerted a synergistic action with GHRH *in vivo*, while *in vitro* merely exhibited additive activity (Smith *et al.* 1997). It was shown that GH-releasing activity of GHS is clearly higher when hypothalamic pituitary axis is intact (Pombo *et al.* 1995, Popovic *et al.* 1995, Hickey *et al.* 1996). These data and finding that the GH response to direct intracerebroventricular GHRP injection was greater than that observed after systemic administration of the same dose (Fairhall *et al.* 1995), point to an important hypothalamic action of GHS.

Somatostatin at a concentration of 10 μ M abolished the effect of both hGHRH and L-585, whereas 5 μ M SS was more effective in suppressing the stimulatory effect of hGHRH. GHS depolarize the plasma membrane of somatotropes by inhibiting K^+ channels, and can act as a functional antagonist of somatostatin that causes somatotropes hyperpolarization (Smith *et al.* 1993, 1997). This functional antagonism between GHS and SS can explain while lower dose of SS did not abolish effect of L-585.

Our results suggest that both extracellular and intracellular calcium are involved in stimulatory effect of L-585 on cultured porcine somatotropes. The depletion of extracellular calcium greatly diminished the stimulatory effect of L-585 suggesting that Ca^{2+} influx has a major contribution in the action of L-585. T- and L-types voltage-gated Ca^{2+} channels are present on somatotropes membrane (Chen *et al.* 1990). Pretreatment of cultured porcine pituitary cells with nifedipine significantly decreased the effect of L-585, indicating an involvement of L-type Ca^{2+} channels in calcium influx induced by L-585. In zero sodium HEPES stimulatory effect of L-585 was greatly attenuated, suggesting that L-585 can depolarize somatotropes membrane by acting on sodium channels. It is possible that K^{+} channels are also involved in somatotropes depolarization since electrophysiology studies showed that peptidomimetics blocked K^{+} currents in somatotropes that lead to depolarization and Ca^{2+} entry through voltage gated channels (Smith *et al.* 1997). Pretreatment of cultures with low calcium HEPES, nifedipine and zero sodium HEPES, almost abolished the second, sustained phase of calcium transient induced by L-585. These results implied that plateau phase of calcium transient evoked by L-585 is result of calcium influx, while the first initial increase in $[\text{Ca}^{2+}]_i$ is result of intracellular Ca^{2+} mobilization. In isolated rat somatotropes GHRP-6 evoked a transient increase in $[\text{Ca}^{2+}]_i$, followed by second long-lasting phase (Herrington & Hille 1994). The first phase was due to calcium release, because a Ca^{2+} channel blocker did not block it, and the second phase was due to calcium influx. It is proposed that GHRPs first mobilize intracellular Ca^{2+} and then Ca^{2+} influx, as a result of membrane depolarization and the action of second messengers on Ca^{2+} channel proteins (Chen *et al.* 1996). We obtained a similar response with L-585 on cultured porcine pituitary cells, suggesting a similar mechanism of action between peptidomimetics and L-585. This agrees with the finding that peptidomimetics and nonpeptidyl GH secretagogues bind to the same receptors on somatotropes (Smith *et al.* 1997).

Results from our experiments where the stimulatory effects of GHRH and L-585 were decreased with SQ, an AC inhibitor, suggest that action of both ligands is mediated through cAMP/PKA pathway. It is well established that GHRH activates the cAMP/PKA pathway in somatotropes (Harwood *et al.* 1984, Frohman *et al.* 1992), and that part of the effect of SS is via its inhibition on cAMP formation (Schonbrunn 1990). The activation of AC by GHS is

controversial. It was observed that GHRP-6 and GHRP-1 did not have direct effect on intracellular cAMP levels in rat and ovine somatotropes (Cheng *et al.* 1989, Akman *et al.* 1993, Wu *et al.* 1996). GHRP-2 increased intracellular cAMP levels in ovine somatotropes, but not in rat somatotropes, suggesting the existence of several subtypes of GHS-R that are differentially expressed in different species (Wu *et al.* 1996, Chen 2000). It seems that L-585 in porcine somatotropes acts on the same subtype of GHS-R that is involved in action of GHRP-2 in ovine somatotropes, because our results indicated the involvement of a AC signaling pathway in the L-585 action on cultured porcine somatotropes.

Results from our study suggest that the action of GHRH and L-585, is also mediated through PLC pathways. The stimulatory effects of hGHRH and L-585 on calcium transient in porcine somatotropes were decreased by pretreatment of cultures with U73122, a PLC inhibitor. Previously, it was suggested that action of GHRP and a non-peptidergic analogue on GH release is mainly mediated by PLC pathway (Cheng *et al.* 1991, 1993). This pathway produces both IP_3 , which leads to release of Ca^{2+} from intracellular stores, and diacylglycerol (DAG), which activates PKC. Consistent with activation of PLC pathway were finding that GHRP-6 and non-peptidergic GHS increased phosphatidylinositol (PI) turnover, and caused translocation of PKC (Adams *et al.* 1995, Mau *et al.* 1995). The cells in our study, whose response to GHRH was decreased in the presence of U73122, probably were LD cells. Low-(LD) and high-density (HD) cells are two morphologically and functionally distinct somatotrope subpopulations described in rats and pigs (Lindstrom & Savendahl 1996, Ramirez *et al.* 1998, 1999). It was shown that GHRH activated AC in both LD and HD subpopulation of porcine somatotropes, whereas GHRH activated PLC pathway only in LD cells (Ramirez *et al.* 1999).

In the past five years, Dr. Jena and his group focused their studies on the molecular mechanism of secretory fusion vesicle. Atomic force microscopy (AFM) in combination with electron microscopy, confocal microscopy, electrophysiology, and molecular and biochemical approaches has been applied to study the molecular mechanism of membrane fusion (Schneider *et al.* 1997, Jena *et al.* 1997, Jeong *et al.* 1999, Cho *et al.* 2002a,b). The AFM is a useful technique for imaging the surface of living cells in three dimensions. The surface of the sample is scanning with pyramidal silicon or silicone nitride tip mounted on a

cantilever spring and deflection of the tip is detected by laser photometry. With AFM studies, new insights have been gained on the structure-function relationships of whole cells, subcellular organelles and macromolecules. According to the commonly accepted theory, the final step in exocytosis is the total incorporation of secretory vesicle membrane at the cell plasma membrane that is later followed by compensatory retrieval of excess membrane by endocytosis. However, studies using electron microscopy demonstrate that following stimulation of secretion there is no loss of secretory vesicles following exocytosis, but depletion of vesicular content results in the formation of empty or partially empty vesicles (Lawson *et al.* 1975, Cho *et al.* 2002b). In addition, earlier electrophysiological measurements on live secretory cells suggested the presence of fusion pores at the plasma membrane where secretory vesicles fuse to release vesicular contents (Alvarez de Toledo *et al.* 1993, Monck *et al.* 1995). Recent studies using AFM demonstrate for the first time the presence of the fusion pore involved in exocytosis in live pancreatic acinar and chromaffin cells (Schneider *et al.* 1997, Cho *et al.* 2002b,c, Lee *et al.* 2002). These studies have shown that "pits" and "depressions" are sites at the apical plasma membrane in live cells, where membrane-bound secretory vesicles transiently dock and fuse to release vesicular contents. Fusion pores located at the apical plasma membrane of pancreatic acinar cells dilate only 25-35 % during exocytosis, showing that there is no total incorporation of secretory vesicles at the fusion pore which would distend the structure much more than what is observed (Schneider *et al.* 1997, Cho *et al.* 2002b,d, Lee *et al.* 2002). These earlier results prompted the current study to determine secretory vesicle dynamics in live GH-secreting cells. First, the stimulatory effect of L-585 on GH secretion from isolated porcine somatotropes was confirmed by RHPA. Surface of plaque around somatotropes stimulated by L-585 was increased, comparing to plaque in resting somatotropes, showing an increase in GH secretion in stimulated cells. Further, plasma membrane of live and fixed GH secreting cells were imaged using AFM, and there was no detectable changes following fixation. Specific plasma membrane structures 'pits' and 'depressions' are present at resting somatotropes. After cell exposure to L-585 40 % increase in the size of 'depressions' was observed, suggesting involvement of these structures in hormone release. This was further confirmed by binding gold-tagged GH antibody to the 'pit' structures in the L-585 stimulated cells. This study for

the first time documented presence of 'pits' and 'depressions' on neuroendocrine cells, suggesting that these structures may be common for all secretory cells.

Perfusion of isolated porcine somatotropes with an endogenous ligand for GHS-R – ghrelin also evoked dose dependent increase in $[Ca^{2+}]_i$ followed by sustained decline above base line. 1 μ M ghrelin increased $[Ca^{2+}]_i$ in 98 % cells that first responded to the hGHRH. Application of ghrelin evoked a similar increase in $[Ca^{2+}]_i$ as a $10 \times$ greater concentration of hGHRH, suggesting that ghrelin is more effective. Similarly, in humans *iv* administration of ghrelin induced prompt increase in circulating GH levels that was clearly higher than the observed after GHRH administration (Takaya *et al.* 2000, Arvat *et al.* 2001). There was no heterologous desensitization between hGHRH and ghrelin, but homologous desensitization occurred after repeated ghrelin application. Previous studies on rat somatotropes have shown that serial ghrelin stimulation at 1 h intervals strongly desensitized somatotropes (Yamazaki *et al.* 2002). In agreement with our results from experiments with L-585, simultaneous application of hGHRH and ghrelin did not have an additive effect on intracellular calcium concentration in cultured porcine somatotropes. It was previously shown *in vivo* studies in humans that ghrelin synergized with GHRH (Arvat *et al.* 2001, Hataya *et al.* 2001), while in *in vitro* studies in rats simultaneous application of these two agents did not elicit either a synergistic or an additive GH response (Yamazaki *et al.* 2002). The similar results were obtained in studies with exogenous GHS suggesting that hypothalamus is important site of action of both endogenous and exogenous GHS and that for their maximal effect an intact hypothalamo-pituitary axis is necessary.

The stimulatory effect of ghrelin on porcine somatotropes was mediated by the GHS-R. In the presence of a putative competitive GHS-R antagonist, (D-Lys³)-GHRP-6, the stimulatory effect of ghrelin was considerably decreased, but not abolished. In a stable CHO cell line expressing rat GHS-R, the $[Ca^{2+}]_i$ increase evoked by small dose of ghrelin was depressed in the presence of 10^{-4} M [D-lys-3]-GHRP-6, but a high dose of ghrelin increased $[Ca^{2+}]_i$ to the level observed in the absence of the antagonist (Kojima *et al.* 1999). This indicated that ghrelin is competitively inhibited by the antagonist.

In the presence of SS and NPY, increase in $[Ca^{2+}]_i$ evoked by ghrelin was significantly smaller. The inhibitory effect of SS can be mediated by decreased cAMP

formation (Chen *et al.* 1996), or by decreased Ca^{2+} influx as a result of an increase in K^{+} conductance and hyperpolarization of somatotropes (Lussier *et al.* 1991). Although SS inhibits the stimulatory effect of GHS on GH secretion from pituitary (Badger *et al.* 1984, Bowers *et al.* 1984, Akman *et al.* 1993), there is evidence suggesting that GHS could act by antagonizing the inhibitory activity of SS on GH release by counteracting its hyperpolarizing effect on somatotrope cell membrane (Goth *et al.* 1992, Smith *et al.* 1997). NPY also exerts a negative influence on GH secretion possibly through either stimulating SS neurons or inhibiting GHRH neurons (Kamegai *et al.* 1998). It was shown that NPY releases SS from median eminence fragments *in vitro* (Rettori *et al.* 1990). NPY neurons express Fos in response to GH and NPY neurons in the arcuate express GH receptors suggesting that NPY neurons play a physiological role in regulation of GH secretion (Chan *et al.* 1996). In the present study we have demonstrated an inhibitory effect of NPY on ghrelin induce increase in $[\text{Ca}^{2+}]_i$ in somatotropes. The inhibitory effect of NPY at pituitary level was previously observed with human somatotropic tumors where NPY reduced GHRH stimulatory effect (Adams *et al.* 1987). In our experiments NPY decreased basal $[\text{Ca}^{2+}]_i$ implying that NPY can reduces stimulatory effect of GHRH and/or GHS by affecting $[\text{Ca}^{2+}]_i$. To reveal the exact role of ghrelin in GH secretion, future studies should focus on the interactions between ghrelin and GHRH, as well as SS, NPY, GH and IGF-I.

Endogenous ligand for GHS-R – ghrelin and nonpeptidyl GHS – L585 produced very similar calcium transient in isolated porcine somatotropes suggesting similar mechanism of action. In low calcium HEPES, in nifedipine or in zero sodium HEPES, the second sustained phase of calcium transient induced by ghrelin was almost completely abolished. These results indicate that the first phase of ghrelin-evoked calcium increase resulted from intracellular Ca^{2+} mobilization, whereas the second phase was consequence of membrane depolarization and calcium influx through voltage dependent calcium channels. Experiments with SQ, an AC inhibitor, and U73122, a PLC inhibitor, have shown that ghrelin mobilized Ca^{2+} from intracellular stores through activation of AC and PLC pathways. We have drawn the same conclusions from studies examining the ability of L-585 to affect $[\text{Ca}^{2+}]_i$ in porcine somatotropes implying that ghrelin and L-585 indeed possess similar mechanisms of action. The similar action of ghrelin and peptidyl GHS has been previously shown (Arvat *et al.*

2001, Bowers 2001), but in the present study, we have for the first time shown a similar action between ghrelin and the nonpeptidyl GHS, L-585.

It is proposed that in somatotropes at least two functional receptors for GHRP exist, GHRP-R1 and GHRP-R2. Binding of GHRP to those different receptors can activate both adenylyl cyclase and phospholipase C, and G_s and G_q are most likely involved in the response (Koch *et al.* 1988). In our porcine pituitary cell cultures, L-585 and ghrelin activated these signal transduction pathways, AC and PLC, therefore they also may act through different types of receptors that are coupled to G_s and G_q (Fig.1). Furthermore, it is possible that cross talk between different signal transduction pathways exists. It was previously demonstrated that GHRP-2 activate PKC and cAMP/PKA pathways in ovine somatotropes and it was proposed that activation of PKC could potentiate the PKA pathway by increasing activity of AC, by enhancing the ability of cAMP to activate PKA or by increasing the size of the readily releasable pool of GH (Chen 2000). In many endocrine cells, such as pancreatic β -cells (Tamagawa *et al.* 1985), these two signal transduction pathways frequently act in concert to induce hormone secretion. It is reasonable to assume that various combinations of the two signaling systems may cooperate and intensify GH secretion from somatotropes. Activation of these different signal transduction pathways result with calcium mobilization from internal stores during the first phase of L-585 and ghrelin induced increase in $[Ca^{2+}]_i$. This initial increase in $[Ca^{2+}]_i$ is followed by second prolonged phase due to calcium influx, that results from somatotrope depolarization by acting on Na^+ and K^+ channels and the activation of calcium channels by acting on different second messengers.

Experiments where ghrelin and L-585 were applied in succession or simultaneously have indicated antagonistic effects between these two agonistes. Application of L-585 10 min after ghrelin did not have additive effect on $[Ca^{2+}]_i$. In addition, the response evoked by L-585 was greatly attenuated when applied in the presence of small doze of ghrelin, that alone did not have any effect on $[Ca^{2+}]_i$. When ghrelin was applied 10 min after L-585 instead of an increase, an abrupt decrease in $[Ca^{2+}]_i$ occurred. These antagonistic effects between ghrelin and L-585 may be result of competition for binding site at GHS-R on somatotropes, which may involve different subtypes of GHS-R (Chen 2000). It is also possible that competition between ghrelin and L-585 may occur downstream from the GHS-R or sub-types.

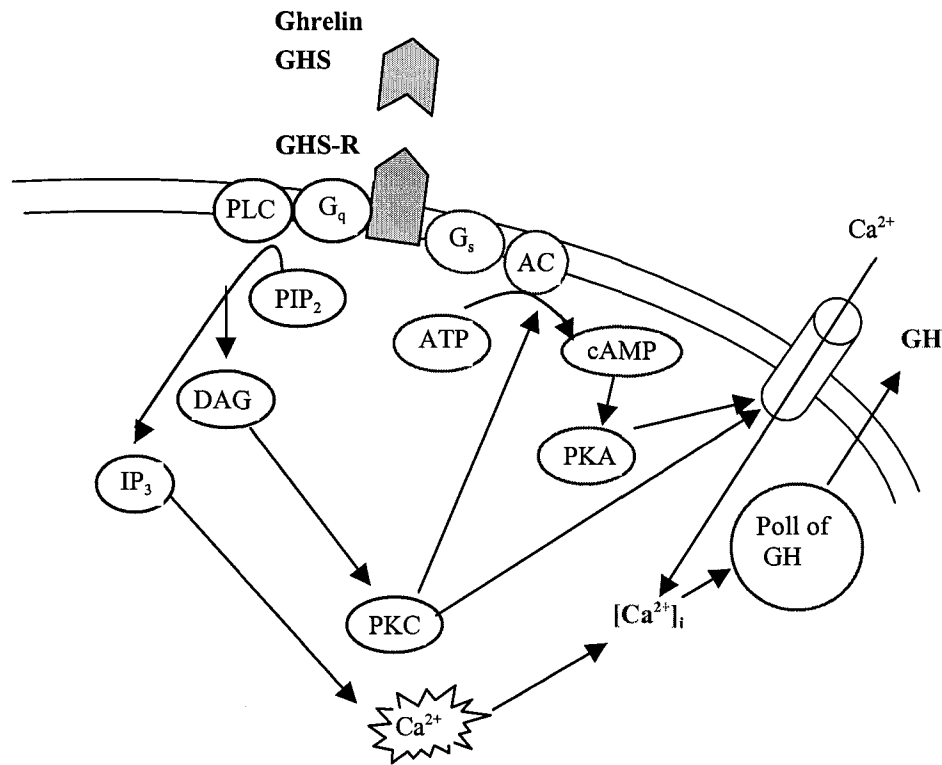


Figure 1 The proposed signalling pathways for ghrelin and GHS in porcine somatotropes. The binding of ghrelin and GHS to a GHS-R activates the PLC and AC pathways via different G-proteins leading to an increase in IP_3 and the activity of PKC and PKA. IP_3 then releases Ca^{2+} from the IP_3 -sensitive Ca^{2+} stores and protein kinases phosphorylate ion channels. These events together result with an increase in $[Ca^{2+}]_i$ and GH secretion (modified from Chen *et al.* 1996).

In summary, L-585 and ghrelin have a direct, dose dependent stimulatory effect on porcine somatotropes. Calcium is mobilized from internal stores during the first phase of calcium transient evoked by ghrelin and GHS, whereas a second prolonged phase results from calcium influx through an increase in cell membrane Ca^{2+} permeability. The latter is reflecting membrane depolarization and action of second messengers on Ca^{2+} channels. Ghrelin and L-585 exerted an antagonistic effect on calcium transients in porcine somatotropes, but the exact basis for these actions is unknown. The full elucidation of the mechanisms by which ghrelin and other specific GHS stimulate GH secretion would gain a

greater insight into our understanding of the mechanisms involved in the regulation of GH secretion and somatic growth. This knowledge may ultimately be applied to production of high quality meat by optimizing GH secretion using pharmaceutical manipulations, or by management, nutrition and/or breeding/genetics. In addition, the knowledge of cellular mechanism of GHS action opens up possibility of clinical use to improve diagnosis and treatment of different disease states associated with altered GH secretion such as GH deficiency, aging, obesity, osteoporosis or cardiovascular diseases.

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APPENDIX A. GLOSSARY OF TERMS

AC - adenylyl cyclase

ACTH - adrenocorticotropin hormone

AFM - atomic force microscopy

AGRP - agouti-related protein

ANOVA - one-way analysis of variance

AP - anterior pituitary

ARC - hypothalamic arcuate nucleus

BSA – bovine serum albumin

Ca^{2+} - calcium

$[\text{Ca}^{2+}]_i$ - intracellular calcium concentration

$\Delta[\text{Ca}^{2+}]_i$ - changes in intracellular calcium concentration

cAMP - cyclic adenosine monophosphate

CART - cocaine and amphetamine-regulated transcript

CHO - Chinese hamster ovary

CNS - central nervous system

DAG - 1-Oleoyl-2-acetyl-sn-glycerol

DMEM - Dulbecco's modified Eagle's medium

EBSS - Earle's balanced salt solution

EGTA - ethyleneglycol-bis-(β -aminoethyl ether) N,N,N',N'-tetraacetic acid

FBS – fetal bovine serum

GH - growth hormone

GHRH - growth hormone releasing hormone

hGHRH - human growth hormone releasing hormone

GHRP- growth hormone releasing peptides

GHS - growth hormone secretagogues

GHS-R - growth hormone secretagogues receptor

HD - high density

HEPES - (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])

HS - horse serum

ICC - immunocytochemistry

icv - intracerebroventricular

IGF-I - insulin-like growth factor I

IP₃ - inositol 1,4,5-trisphosphate

iv - intravenous

K⁺ - potassium

L - 692,585 – L-585

LD - low density

MEM - minimum essential medium

MPA - the mean plaque area

Na⁺ - sodium

NEFA – nonesterified fatty acids

NPY – neuropeptide Y

NSF - N-ethylmaleimide-sensitive fusion protein

PBS – phosphate buffer saline

PFC - plaque forming cells

PI - phosphatidylinositol

PKA - protein kinase A

PKC - protein kinase C

PLC - phospholipase C

POMC – proopiomelanocortin

PRL – prolactin

PVN - hypothalamic periventricular nucleus

RHPA - reverse hemolytic plaque assay

S.E. - standard error

S.E.M. - standard error of mean

SNAPs - soluble N-ethylmaleimide-sensitive fusion protein attachment proteins

SNAREs - SNAP receptors

SP - substance P

SS; SRIH – somatostatin

TM – transmembrane

TSI - total secretion index

VMN - hypothalamic ventromedial nucleus

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